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CHOLINE-CONTAINING ACETAL PHOSPHATIDE AND THIOBARBITURIC ACID REACTION IN THE RAT LIVER*

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Kohn and Liversedge (1) found the very interesting fact that aerobic incubation of animal tissues frequently results in the production of a compound which yields red chromogen on heating with thiobarbituric acid (TBA). In the case of proliferating tissues such as regenerating rat liver and rabbit bone marrow where active cell division takes place, the TBA-reaction is generally very weak (2, 3), and particularly, almost lost in tumor tissues (4, 5, 6). Therefore, it was thought that some light might be thrown on the specificity of tumor tissues through studying the nature of TBA-reacting substance occurring in normal tissues. In a previous paper (6), we have shown that naturally occurring TBA-reacting substance of the rat liver is solely confined to a phospholipid fraction and readily absorbed on MgO in methanol solution. It was also suggested that the deficiency of TBA-reaction in tumor tissues may probably be due to some qualitative modification of a certain kind of phospholipid or its complete absence from the tumor.

In the present study, a substance responsible for TBA-reaction was isolated from the phospholipid fraction obtained from the rat liver and identified as choline-containing acetal phosphatide (plasmalogen).

Preliminary Experiments

Extraction with Ethanol Livers of male rats were used throughout as material. First, ethanol-soluble and ethanol-insoluble fractions prepared according to the method of Folch (7, 8) were respectively examined for the TBA-reaction. 1.6 g of rat liver was homogenized in 8 ml of acetone and centrifuged. The precipitate was washed by suspending it in 8 ml of acetone and centrifuged. The washed precipitate thus obtained was extracted with 8 ml of ethanol. After centrifugation, the supernatant was filtered and the volume of filtrate was adjusted to 20 ml by the addition of ether (Fraction 1a). 9.5 ml of the Fraction 1a was evaporated on a boiling water bath, and dissolved in 19 ml of methanol (Fraction 2a). Since it has been reported by Hevesy (9) that choline phospholipid is not adsorbed on MgO in methanol solution, 1 g of MgO was added to 18 ml of the Fraction 2a and after shaking for 20 minutes, MgO was remorved by

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centrifugation and the supernatant containing MgO-unadsorptive material was obtained (Fraction 3a).

The precipitate remaining after ethanol extraction was then extracted twice with 8 ml of petroleum ether, and the combined petroleum ether extract evaporated on the boiling water bath. The dried material obtained was dissolved in 5 ml of ether and filtered, and the filtrate concentrated to about 1 ml, then 7 ml of ethanol and 1 drop of $MgCl_2$-saturated ethanol were added, and centrifuged. The precipitate was dissolved in 20 ml of ether (Fraction 4a : so-called cephalin fraction), and the supernatant was made to 20 ml by the addition of ether (Fraction 5a). The residue remaining after petroleum ether extraction was dried and homogenized in 80 ml of 1/15 M phosphate buffer, pH 5.9 (Residue fraction).

0.5 ml of the Fractions 1a, 4a and 1 ml of the Fractions 2a and 3a were respectively evaporated to dryness on the boiling water bath, and to each of these dried Fractions (equivalent to 40 mg of original liver) was added 2 ml of Residue fraction (also equivalent to 40 mg of liver) and incubated aerobically at 38°C with shaking for 1 hour. At the end of the incubation period, 2 ml of 20 per cent trichloroacetic acid and 4 ml of 0.67 per cent TBA were added, and then the mixture was placed in the boiling water bath for 15 minutes. The pink color appeared in the course of boiling. After cooling and centrifugation, the optical density of the pink color produced in the supernatant was measured in a Hitachi photometer with a No. 50 filter, and corrected against the optical density of Residue fraction alone. The results are expressed as (-Log T) $\times 100$.

Table 1. TBA value for ethanol extract and petroleum ether extract

No. of exp.	Etranol extract			Petroleum ether extract	
	Fraction 1a	Methanol-soluble		Ethanol-sol. Fraction 4a	Ethanol-insol. Fraction 5a
		Fraction 2a	MgO-unadsorptive. Fraction 3a		
1	16.0	12.2	1.3	0.2	0.0
2	26.0	12.7	0.3	0.1	1.0
Average	21.0	12.5	0.8	0.2	0.8

As shown in Table 1, TBA-reacting substance was found to be in ethanol-soluble fraction (Fraction 1a), but not in ethanol-insolube and petroleum ether-soluble fraction (Fraction 4a and 5a). Of ethanol-soluble fractions, the methanol-soluble fraction (Fraction 2a) was most active as regards TBA-reaction, the methanol-soluble and MgO-unadsorptive fraction (Fraction 3a) being however inactive. It is concluded therefore that the TBA-reacting substance must be soluble in both ethanol and methanol, and readily adsorbed on MgO in methanol solution.

Extraction with Acetone The possibility that some quantities of the TAB-reacting

substance may have been lost during the course of acetone extraction adopted in the first step of the previous procedure can not be excluded. Therefore, the TBA values for acetone extract and for ethanol extract were then compared.

1.6 g of liver was homogenized in acetone and centrifuged. The supernatant was made to 8 ml with acetone (acetone extract). The precipitate was extracted with ethanol and, after centrifuging and filtering, the volume of filtrate was adjusted to 8 ml (ethanol extract). The residue which had remained after ethanol extraction was washed with petroleum ether, dried, and homogenized with 80 ml of 1/15 M phosphate buffer, pH 5.9 (Residue fraction). 0.2 ml of acetone extract and ethanol extract respectively were evaporated to dryness on a boiling water bath, and 2 ml of Residue fraction were added to each sample and incubated. The TBA values were measured as described previously, and the results are shown in Table 2. The TBA value is higher in ethanol extract than in acetone extract, but a considerable amount of the TAB-reacting substance appears to have been lost during acetone extraction, probably because a certain proportion of the phospholipids is readily extractable with acetone. At any rate, it may be said that much of the TBA-reacting activity is confined in the ethanol extract. Thus, a further fractionation of the ethanol extract was carried out in the following experiments.

Table 2. TBA value for acetone extract and ethanol extract

No. of experiment	Acetone extract	Ethanol extract
1	3.1	12.0
2	13.1	17.4
3	8.0	9.6
4	9.1	11.0
Average	8.3	12.5

Further Fractionation of Ethanol Extract The ethanol extract and Residue fraction were prepared as described previously. In this series of experiments, however, the end volume of ethanol extract was made to 8 ml (Fraction 1b), so that 0.2 ml of ethanol extract was equivalent to 40 mg of liver. To 4.8 ml of Fraction 1b, 1 ml of aqueous 50 per cent $CdCl_2$ was added, and, after standing for 1 hour it was centrifuged, and 5 ml of chloroform was added to the supernatant. Then $CdCl_2$ was removed by extracting the chloroform solution with 30 per cent ethanol twice, and the volume of chloroform solution was adjusted to 12 ml (Fraction 2b). 11.5 ml of Fraction 2b was concentrated to about 1 ml, and 7 ml of acetone and 3 drops of $MgCl_2$-saturated ethanol were added and centrifuged down. The precipitate thus obtained was dissolved in 11.5 ml of ether (Fraction 3b), and the supernatant was made to just 11.5 ml by addition of ether (Fraction 4b). 0.2 ml of Fraction 1b, 0.5 ml of Fractions 2b, 3b and 4b, were evaporated to dryness, and then 2 ml of the Residue fraction was added to each. After incubation, the TBA value was measured as usual.

As shown in Table 3, the TBA-reacting substance contained in ethanol extract is not precipitated with $CdCl_2$ and extracted from $CdCl_2$-treated ethanol fraction with

Table 3. TBA values for various fractions from ethanol extract

No. of experiment	Ethanol extract			
	Fraction 1b	Non-precipitable with $CdCl_2$ and chloroform extract		
		Fraction 2b	Acetone-insoluble Fraction 3b	Acetone-solulbe Fraction 4b
1	21.6	24.2	5.5	11.3
2	19.3	16.3	5.2	16.2
3	12.0	18.0	2.0	10.3
Average	17.6	19.5	4.2	12.6

Table 4. TBA values for various fractions from ethanol extract

No. of experiment	Ethanol extract			
	Fraction 1b	Non-precipitable with $CdCl_2$ and chloroform extract		
		Fraction 2b	Acetone-insoloble Fraction 3c	Acetone-soluble Fraction 4c
1	12.0	18.0	6.0	1.7
2	17.4	12.6	10.0	1.0
3	21.0	15.0	11.4	0.8
Average	16.8	15.2	9.1	1.2

chloroform. It is also obvious that this substance is hardly precipitable from chloroform solution with acetone.

Secondly, a similar experiment was carried out, but, in this case, 11.5 ml of chloroform fraction was evaporated to dryness and this dried material was suspended in 1 ml of petroleum ether, then 7 ml of acetone and 3 drops of $MgCl_2$-saturated ethanol were added and centrifuged. The precipitate was dissolved in 11.5 ml of ether (Fraction 3c), and the supernatant was adjusted to 11.5 ml by addition of ether (Fraction 4c). Thus, in this case, acetone had been added to petroleum ether solution containing the TBA-reacting substance, not to chloroform solution. Table 4 shows that the TBA value is far higher in Fraction 3c than in Fraction 4c. It is interesting to find that the TBA-reacting substance is precipitated from petroleum ether solution with acetone, but not from chroloform solution with acetone.

Isolation of TBA-Reacting Substance from the Phospholipid Fraction

From the results of the preliminary experiments it is obvious that the TBA-reacting substance is soluble in ethanol, not precipitated with $CdCl_2$, and can be transferred to chloroform layer from $CdCl_2$-treated ethanol extract. It is also precipitated from a petroleum ether solution with acetone. A considerable amount of TBA-reacting substance, however, appears to be lost with acetone extraction at the first step of the

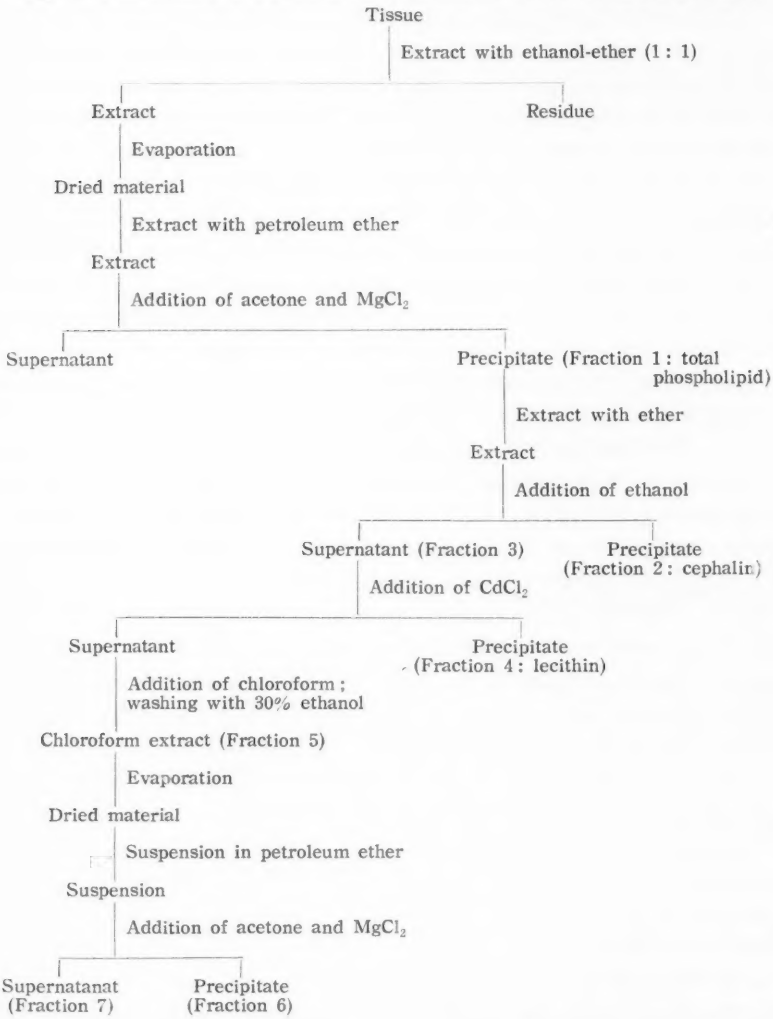
procedures antecedent to ethanol extraction. In the previous paper (6), it has been shown that a phospholipid fraction which is prepared by the method of Bloor (10) is the only fraction capable of reacting with TBA and that a higher TBA value is obtained in the phospholipid fraction than in homogenates. Making use of this method of extraction, a fairly quantitative isolation of TBA-reacting substance from the rat liver phospholipid was made possible as follows:

1.6 g of liver was homogenized in 10 ml of ethanol-ether (1 : 1) and the homogenate was immersed in a boiling water bath for about 1 minute, then re-homogenized and centrifuged. The precipitate was washed with about 10 ml of ethanol-ether (1 : 1) and centrifuged. The precipitate thus obtained was homogenized in 80 ml of 1/15 M phosphate buffer, pH 5.9 (Residue fraction). Combined ethanol-ether extract was evaporated to dryness and the dried material was extracted with petroleum ether three times. After filtering, petroleum ether extract was concentrated to about 2 ml, and 14 ml of acetone and 1 drop of $MgCl_2$-saturated ethanol were then added. After standing for 10 minutes and centrifuging, the precipitate was dissolved in 8 ml of ether (Fraction 1). This fraction contains the total phospholipid. 7.8 ml of Fraction 1 was concentrated to 2 ml, and 10 ml of ethanol was added and centrifuged. The precipitate was dissolved in 7.8 ml of ether (Fraction 2: so-called cephalin fraction), and the volume of supernatant was adjusted to 15.6 ml by the addition of ether (Fraction 3). Then 15.2 ml of Fraction 3 was concentrated to 5 ml, 1 ml of 50 per cent $CdCl_2$ was added and centrifuged. The precipitate was dissolved in 7.6 ml of chloroform (Fraction 4: lecithin fraction). To the supernatant, 5 ml of chloroform was added, and $CdCl_2$ was removed by extracting the chloroform solution with 30 per cent ethanol four times. After centrifugation, the volume of chloroform solution was adjusted to 7.6 ml (Fraction 5). 7.4 ml of Fraction 5 was completely evaporated to dryness and the dried material was suspended in 1 ml of petroleum ether, and then 7 ml of acetone and 3 drops of $MgCl_2$-saturated ethanol were added and centrifuged. The precipitate was dissolved in 18.5 ml of ether (Fraction 6), and the supernatant was made to 18.5 ml with ether (Fraction 7). These fractionation procedures are illustrated in Figure 1.

0.2 ml of Fractions 1, 2, 4 and 5, 0.4 ml of Fraction 3 and 0.5 ml of Fractions 6 and 7 were evaporated to dryness respectively (each equivalent to 40 mg of liver), and then 2 ml of Residue fraction (also equivalent to 40 mg of liver) was added to each sample. The TBA valeus were then measured as described above. The results are shown in Table 5.

A high TBA value similar to that for Fraction 1 is seen for Fractions 3, 5 and 6, and not for Fractions 2, 4 and 7. Since, starting from the fraction of total phospholipid, cephalin which is insoluble in ethanol has been removed with Fraction 2, and lecithin and ethanolamine-containing acetal phosphatide (plasmalogen) which are

Fig. 1. Fractionation of the tissue for obtaining the TBA-reacting phospholipid.



precipitated with $CdCl_2$ removed with Fraction 4, it seems that Fraction 6 contains a new phospholipid hitherto unknown. It is also very interesting to observe that this new phospholipid fraction shows a strong TBA-reaction, indicative of the formation of a peroxide of lipid, whereas cephalin, lecithin and so-called acetal phosphatide fractions are almost inactive as regards the TBA test. As the TBA value for total phospholipid is 24.3 and that for Fraction 6 is 16.4, a full recovery is not attained by these procedures, but a considerable proportion of TBA-reacting substance originally

Table 5. TBA values for various fractions separated from phospholipid fraction

No. of experiment	Total phospholipid						
	Fraction 1	Ethanol-insoluble Fraction 2	Ethanol-soluble Fraction 3	Precipitable with $CdCl_2$ Fraction 4	Non-precipitable with $CdCl_2$ and chloroform extract Fraction 5	Acetone-insoluble Fraction 6	Acetone-soluble Fraction 7
1	31.2	6.6	27.3	—	—	24.5	1.3
2	21.8	12.8	20.5	9.5	18.3	8.2	3.4
3	14.0	3.8	13.5	8.1	17.4	12.3	1.0
4	30.2	13.8	27.3	1.7	11.2	10.6	6.8
Average	24.3	9.3	22.2	6.4	15.6	16.4	3.1

contained in total phospholipid appears to pass into Fraction 6.

Analysis of Isolated TBA-Reacting Substace

Determination of Phosphorus and Nitrogen The TBA-reacting substace above described was isolated from 1.6 g of rat liver and dissolved in 16 ml of ethanol-ether (1 : 1). Each 1 ml of this solution was heated in a Kjeldahl flask with 2.5 ml of 10 N H_2SO_4 for 1 hour. After cooling, 3 drops of 30 per cent H_2O_2 were added and heated again for 30 minutes, and this procedure was repeated once more. Then 1 ml of deionized water was added and heated for 10 minutes. One sample thus obtained was used for the measurement of phosphorus content by the method of Lohmann and Jendrassik (11), and the other one for the determination of nitrogen by the micro Kjeldahl method. Then the molar ratio of phosphorus to nitrogen was calculated, and it was found that P : N molar ratio is 1.02, that is, the TBA-reacting substance contains P and N in equi-molar level (Table 6).

Table 6. P : N molar ratio of TBA-reacting substance

No. of sample	P. : N
1	1.05
2	1.03
3	0.97
Average	1.02

Spot Tests using Filter Paper Disk Chromatograms The isolated TBA-reacting substance was dissolved in a small amount of chloroform-methanol (4 : 1) and analyzed by the method of Hack (12). A small amount of the sample was applied to the center of a filter paper (Toyo, No. 7, 5.5 cm), and, after drying, 0.025 ml of chloroform-methanol (4 : 1) was then added not allowing the diameter of the wet spot to exceed 10 mm. Then, after this eluent (chloroform-methanol) had been evaporated, about 0.05 ml of acetone was added to let the wet spot spread to a ring 40 mm in diameter, this last procedure being repeated four times. After drying, methanol was finally added, care being taken to confine the diameter of the wet spot to about 30 mm. Then

the filter paper chromatogram thus obtained was cut into four or five sectors, and each sector was treated with different reagents. For the detection of choline, one sector was immersed in 0.05 M reinecke salt for 2 hours, and other in I_2-KI solution for 1 minute. Pink color with reinecke salt, and yellow color with I_2 will indicate the presence of a choline lipid. In the case of the test for amine lipid, a sector was treated with 0.005 M ninhydrin and dried for 15 minutes at 105°C; lavender ring appears at the site of amine lipid. For acetal phosphatide, another sector was treated with 0.005 M $HgCl_2$ for 1 minute and immersed in 2: 4-dinitrophenylhydrazine dissolved in dilute HCl, or sometimes this was treated with $HgCl_2$ and immersed in in fuchsin-sulphurous acid (Schiff's reagent). A yellow color with 2: 4-dinitrophenylhydrazine and a red color with fuchsin-sulphurous acid indicate the presence of acetal phosphatide.

Table 7. Qualitative analysis of TBA-reacting substance

No of sample	Choline		Amino group	Aldehyde	
	Reinecke salt	I_2	Ninhydrin	2 : 4-dinitro-phenylhydrazine	fuchsin-sulfurous acid
1	+	+	−	+	
2	+	+	−	+	
3	+	+	−	+	+
4	+	+	−	+	+

The results are shown in Table 7. The TBA-reacting substance isolated shows a positive reaction respectively with reinecke salts, I_2, 2: 4-dinitrophenylhydrazine and fuchsin-sulphurous acid, but not with ninhydrin. Thus it is obvious that the TBA-reacting substance contains choline and aldehyde, but not amino group.

From these results it is concluded that the isolated TBA-reacting substance is a phospholipid containing P and N in equi-molar ratio and that it is presumably a choline-containing acetal phosphatide, rather than a well-kown ethanolamine-based acetal phosphatide.

Discussion

The nature of TBA-reacting substance in animal tissues has not been known for certain. Following the discovery by Kohn and Liversedge (1) of the occurrence of TBA-reacting substance in animal tissues, the same color reaction was reported to occur in the cases of pure linolenic acid and other unsaturated fatty acids whether it is in a free state or whether in a phospholipid molecule (13). Then, Wilbur, Bernheim and Shapiro (14) showed that, in contrast with oxidative produced of linolenic acid, the color produced by linoleic is very weak, arachidonic, oleic and stearic acids being inactive under similar conditions. They considered therefore that under physiological conditions the TBA-reaction may be taken primarily as an index of the amount

of oxidized linolenic acid present in animal tissues. But from this point of view, it is difficult to explain the low TBA value found in tumor tissue (4, 5, 6), for linolenic acid contents of tumor tissues are generally high (cf. 4).

In the present study, the TBA-reacting substance contained in normal tissues was isolated and identified as choline-containing acetal phosphatide. Therefore, it seems that the incapability of tumor tissues as regards TBA-reaction is due to a conversion of this acetal phosphatide into a more or less saturated form or its complete disappearence therein. As to the so-called acetal phosphatide in general, its presence in nuclei from Walker carcinoma 256 was reported by Haven (15). Also, Lapp (16) reported that the mitochondria of carcinomatous tissue are very rich in this lipid, and Yarbro and Anderson (17) showed that this lipid might be involved in the active regeneration of rat liver. Since, however, all these determinations were done on the total acetal phosphatide (well-known ethanolamine-based acetal phosphatide plus only rarely reported choline-based), it is difficult from their results to know the actual content of choline-based acetal phosphatide in tumor tissues or in the regenerating liver. As regards the content of choline-containing acetal phosphatide in tumor tissues, further studies are now being carried on by the isolation method described in this paper.

While most workers in lipid chemistry are agreed that ethanolamine-containing acetal phosphatide is predominant in animal tissues, some workers reported the presence of choline-containing acetal phosphatide. Klenk *et al.* (18, 19, 20) claimed to have detected this lipid in heart muscle phospholipid. Rapport and Alonzo (21) obtained choline-containing acetal phosphatide from beef heart lecithin and they proposed to call it phosphatidal choline. According to Hack (12) animal tissues appear to contain two or more acetal phosphatides, one of which being acetone-soluble and therefore probably not phosphatide. Recently it has been shown by Lovern, Olley, Hartree and Mann (22) that there is little or no lecithin found in ram spermatozoa and that the predominant intracellular phospholipid of ram semen is a choline-based acetal phosphatide. The metabolic significance of this lipid, however, has not yet been known. At any rate, it is an interesting phenomenon that, among the many lipids of animal tissues, only the oxidative product of choline-containing acetal phosphatide is capable of reacting with TBA, although the physiological role of this peroxide formation is unknown at the present time.

Summary

A substance which reacts specifically with thiobarbituric acid and produces a red color was isolated from normal rat livers, and it was identified as choline-containing acetal phosphatide. This lipid was soluble in ethanol, not precipitated from ethanol extract with $CdCl_2$, extractable from $CdCl_2$-treated ethanol solution with chloroform,

and precipitated from petroleum ether solution with acetone. Since it has been known that this TBA-reaction is absent from the tissues in which cell proliferation is actively taking place, especially in tumors, the present findings concerning the nature of TBA-reacting substance are not without interest from the viewpoint of cancer research.

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ON THE QUALITATIVE CHANGE IN THE NATURE OF CHOLINE PLASMALOGEN IN TUMOR TISSUES*

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When the homogenate from normal tissues are incubated aerobically, the peroxide of lipid, which on reacting with thiobarbituric acid (TBA) produces a red chromogen, is often formed (1, 2, 3). However, little or no TBA-reaction is found in tumor tissues (3, 4). The TAB-reacting lipid of the rat liver is involved in a phospholipid fraction (5), and it has recently been found that the lipid responsible for the TBA-reaction is choline plasmalogen, that is, choline-containing acetal phosphatide (6). The lack of TAB-reaction in tumor tissues was therefore considered in the previous paper (6) as due to either disappearance of choline plasmalogen in those tissues or a qualitative modification of fatty aldehyde portion of this lipid. The facts presented in this paper show that unsaturated fatty aldehyde portion of choline plasmalogen is altered to a more or less saturated state in tumor tissues. Some properties of choline plasmalogen in normal tissues have also been investigated.

Materials and Methods

Normal rat livers. rat primary hepatomas induced by p-dimethyaminoazobenzene, rat ascites hepatomas (AH7974), and rat fibrosarcomas (Umeda rhodamine sarcomas) were used as material. Ascites hepatoma cells were separated from ascites serum by centrifuging at 800 r.p.m. for 10 minutes, washed with 0.9 per cent NaCl solution and centrifuged at 500 r.p.m. for 10 minutes. The principitated tumor cells were then suspended in a small amount of 0.9 per cent NaCl solution and centrifuged for 10 minutes at 12,000 r.p.m. in a refrigerated centrifuge, and the precipitated, packed cell mass weighed. 1.6 g of these solid tissues or packed ascites hepatoma cells was used for isolation of choline plasmalogen.

The isolation procedure of this lipid has already been reported in the previous paper (6). In this experiment, however, the procedures of evaporation and of concentration to reduce the volume of the solvent at a reduced pressure were all carried out in nitrogen gas atomosphere. For the determination of the choline plasmalogen contents of various tissues, the lipid thus obtained was dissolved in wet ether, centrifuged to remove $MgCl_2$, and the supernatant of ether solution evaporated, dried *in*

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vacuo over $CaCl_2$, and weighed. Iodine number was measured by the method of Yasuda (7). In this experiment, however, iodine number was calculated on the basis of actual weight of lipid, instead of determining the lipid content by the oxidation method of Yasuda.

Results

A choline plasmalogen fraction was isolated from tumor tissues, such as primary hepatoma, ascites hepatoma and fibrosarcoma by the same procedures as was applied to the normal rat liver in the previous study (6). After having confirmed by the spot test on filter paper disk chromatogram (6, 8) that this lipid fraction of tumor also contains choline and aldehyde but not amino group, the contents of this lipid in tumors was compared with that in the normal rat liver.

The figures for the choline plasmalogen content of normal and tumor tissues will be found in Table 1. It will be seen that a considerable amount of choline plasmalogen is present in tumor tissues, too, though slightly less in amounts than in normal rat liver. Thus, there is apparently no difference in the choline plasmalogen contents between the normal and tumor tissues. The question may be raised at once: how is it that tumor tissues show no TBA-reaction in spite of the presence of the lipid? Therefore, an attempt was made to compare the iodine number of choline plasmalogen isolated from tumor tissues with that of lipid from normal rat liver.

Table 1. Choline plasmalogen contents of normal and tumor tissues (mg/g of tissue).

No.	Liver	Primary hepatoma	Ascites hepatoma	Fibrosarcoma
1	14.3	10.7	7.3	13.2
2	11.7	9.8	8.6	10.6
3	16.3	9.8	8.9	14.4
4	12.0	9.9	8.1	
Average	13.6	10.1	8.2	12.7

The data for the iodine number of the choline plasmalogen are given in Table 2.

Table 2. Iodine numbers of choline plasmalogen isolated from normal and tumor tissues.

No.	Liver	Primary hepatoma	Ascites hepatoma	Fibrosarcoma
1	75	35	47	32
2	73	40	46	45
3	75	61	52	47
Average	74	45	48	41

It is clear from this Table that the iodine number of this lipid is definitely lower in

tumor tissues than in normal liver, although some fluctuation is seen in the values for primary hepatoma. Therefore, it seems that choline plasmalogen of tumor has undergone certain qualitative changes in its degree of unsaturation during the course of carcinogenesis and that this less unsaturated state of its fatty aldehyde portion is responsible for the lack of TBA-reaction in that tissue.

Next, the actual TBA values for isolated choline plasmalogen were compared between normal and neoplastic tissues. It has already been shown in the previous paper (5) that the lipid or phospholipid fraction alone cannot give TBA-reaction, and that a cofactor is needed for this reaction. The residue remaining after extraction of total lipid of rat liver (ethanol : ether, 1 : 1) was used as a cofactor in this experiment. 0.5 mg of isolated choline plasmalogen, 15 mg of dried "residue", and 2 ml of 1/15M phosphate buffer (pH 5.9) were mixed and incubated aerobically at 38°C with shaking for 1 hour. After incubation, 2 ml of 20 per cent trichloroacetic acid and 4 ml of 0.67 per cent TAB were added, and then the mixture was placed in a boiling water bath for 15 minutes. After cooling and centrifuging, the optical density of the supernatant was measured in a Hitachi photometer at 500 mμ filter. The results are expressed as $(-\text{Log T})\times 100$ in Table 3. It is true that choline plasmalogen in ascites hepatoma and fibrosarcoma can react with TBA, but it is to be noticed that its TBA value is very low as compared with that for normal liver. These results again indicate that reduced potency to react with TBA of tumor tissues is attributable to some changes in the nature of this lipid, rather than to the presence of some inhibitor or antioxidant in tumor tissues.

Table 3. TBA values for isalated choline plasmalogen.

Tissues	TBA value $=(-\text{LogT})\times 100$
Liver	17.3
Ascites hepatoma	5.9
Fibrosarcoma	5.7

For the sake of comparison, the relative capacity of male rat tissues as regards the TBA reaction was then investigated. The TBA values of 121 for brain, 109 for testis, 100 for liver, 56 for thymus, 51 for kidney and submaxillary gland, 50 for spleen, 47 for adrenal, 39 for epididymis, 23 for lung, 17 for pancreas, 15 for heart muscle, 13 for skeletal muscle, 6 for small intestine and prostate gland, and 5 for seminal vesicle were obtained. It seemed therefore interesting to examine whether the low intensity of TBA-reaction in some normal tissues is due to the low concentration of choline plasmalogen, or whether it is due to qualititative changes in their fatty aldehyde portion as in the case of tumor tissues. Thus the isolation of choline plasmalogen was done using respectively the tissues of lung, heart muscle, and small intestine in rats, and the contents of this lipid and their iodine numbers were compared. Results are shown in Table 4. It will be seen that even tissues showing a relatively low TBA-reaction, such as lung, heart muscle, and small intestine containe contain considerale amounts of plasmalogen as liver does, indicating that low TBA

Table 4. The contents and iodine number of choline plasmalogen in normal tissues showing low TBA values.

Tissues	Contents (mg/g of tissue)	Iodine numbers
Lung	10.3	50
Heart muscle	10.3	76
Small intestine	10.3	51

values for these normal tissues are not due to the deficiency of this lipid. It is also seen that low TBA value is not always the outcome of the low iodine number of choline plasmalogen contained, since the lipid isolated from heart muscle shows a high iodine number in spite of its low capacity as regards the TBA-reaction.

However, in so far as the comparison is made between a tumor and its homologous normal tissue (such as hepatoma and liver), it may be concluded that the nature of choline plasmalogen of tumor differs greatly from that of normal tissues in the degree of unsaturation. This finding seems very important to the author, because such a change in the nature of a specific phosphatide may be regarded as one of the characteristics of tumors in general.

Discussion

Wilbur, Bernheim and Shapiro (9) have shown that, of the pure fatty acids tested, only the autoxidation product of linolenic acid is capable of reacting with TBA, essentially the same color as in the case of animal tissues being thereby produced, while linoleic, arachidonic, oleic and stearic are wholly inactive in this respect. However, the enigma exists that tumor tissues generally contain more free linolenic acid than normal tissues in spite of their low capacity for TBA-reaction (4). The present author has shown in the previous paper (6) that TBA-reaction in the rat liver is due to some autoxidation products of choline plasmalogen but not to those of lecithin and cephalin. It seems probable that TBA-reaction of lecithin and cephalin reported by Bernheim *et al.* (1) is due to a contamination of choline plasmalogen in their preparations. The results reported in this paper show fairly conclusively that fatty aldehyde portion of choline plasmalogen in tumor tissues is undergoing changes in the degree of unsaturation, and that some such changes are responsible for the decreased TBA value in tumor tissues. Since, in animal tissues, free fatty acids are only found at very low concentrations (10), it is likely that some specific phospholipid such as choline plasmalogen may play an important role in tumor metabolism. It is also probable that a qualitative change of this lipid towards the direction of saturation may be connected in some unknown way with the changes in the nature of respiration in tumor, since, according to Medes and Weinhouse (11), fatty acids constitute the main shbstrate for tumor's endogeneous respiration.

Summary

Normal tissues, such as rat liver, lung, heart muscle, and small intestine, were

found to contain considerable amounts of choline plasmalogen. In rat primary hepatoma, ascites hepatoma and fibrosarcoma also, choline plasmalogen is contained in appreciable amounts. However, it was found that the iodine number of this lipid was always very low in the case of these tumor tissues as compared with that of the lipid isolated from the homologous normal tissue, indicating that the lipid is undergoing a profound change in the degree of unsaturation and is no longer capable of showing TBA-reaction.

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INFLUENCE OF HYPOPHYSEAL FACTORS ON LIVER CATALASE ACTIVITY IN NORMAL AND TUMOR-BEARING RATS*

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Since the studies of Greenstein and his coworkers (1, 2, 3) it has been well known that the liver catalase activity in tumor-bearing animals becomes greatly reduced with growth of the tumor. This phenomenon was later extensively studied by Weil-Maherbe and Schade (4) and Hargreaves and Deutsch (5), and according to Price and Greenfield (6), the depression of the catalase activity is due to a lowering of the concentration of the enzyme. Experiments carried out by Nakahara and Fukuoka (7, 8, 9, 10, 11, 12) have indicated further that all tumors produce a specific substance (toxohormone) capable of markedly depressing liver catalase activity in mice under *in vivo* conditions.

On the other hand, the liver catalase level appears to be connected in some way with the hormonal conditions in animals. For example, under normal conditions, the liver catalase activity in male animals is always higher than in female according to Hargreaves and Deutsch (5) and Adams (13, 14). Also, adrenalectomy or orchioectomy was reported to cause a decrease of liver catalase activity, whether in mice (13, 14, 15) or in rats (16). Moreover, it was reported by Gaebler and Mathies (17) that hypophysectomy caused an increase in liver catalase activity, and according to Zeckwer *et al.* (18), a similar effect was produced by thyroidectomy. (As regards the relationship between tumor and host animals, see a review recently published by Begg (19).)

In the present study, an attempt was made to examine the possible interrelations between hypophyseal factors and liver catalase level in tumor-bearing animals. It was found that tumor exerts its influence on liver catalase through hypophysis or with the aid of some of the hypophyseal hormones, and that the growth hormone of the anterior pituitary plays an important role in the depression of liver catalase.

Materials and Methods

Male and female albino rats weighing from 150 to 250 g were used as material throughout the experiments. Hypophysectomy was performed through the parapharyn-

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geal approach under ether anesthesia by the methods of Takewaki (20) and Selye (21) slightly modified. Hypophysectomized rats which showed a cessation of body weight increase at 2 weeks after operation were used, completeness of operation being confirmed at autopsy by examining the atrophy of pituitary target organs, especially that of testis and adrenals.

Rats bearing fibrosarcoma (Umeda rhodamine sarcoma) which had grown for 2 to 3 weeks after being subcutaneously inoculated was used as test animals.

As a crude pituitary preparation, acetone dried powder of beef anterior pituitary was prepared by placing the minced tissue directly into 20 volumes of chilled acetone, changing acetone two times, and after being dried in a vacuum desiccator. Dried material thus obtained was then pulverized in a mortar, and injected into animals intraperitoneally in saline suspension, in a dose of 10 mg, 25 mg, and 50 mg per animal per day respectively for 5 days.

A pure pituitary hormone, growth hormone (U. S. P.), was also administered intraperitoneally in a daily dose of 0.2 mg for 5 days or 30 days in an alkaline saline solution. This dose was found to bring about body weight increase in both normal and hypophysectomized rats.

For the estimation of catalase activity, two methods were adopted, one being the iodometric titration and the other the manometric method. Details of the methods are as follows.

Animals were sacrificed by decapitation and livers removed were immediately placed in cold saline. Before weighing, the liver lobe was blotted on a filter paper. Iodometric titration was carried out using whole homogenates by the method of Herbert (22) with some modification; 50 mg of liver were homogenized in 10 ml of 0.01 M of phosphate buffer, pH 7.0. To 0.5 ml of this homogenate which contained 2.5 mg of liver tissues were added 2.0 ml of 0.005 M hydrogen peroxide diluted with 0.01 M phosphate buffer (pH 7.0) in an ice bath. After 10 seconds the reaction was stopped by the addition of 3.0 ml of the mixture of 2 N H_2SO_4 and 5 per cent trichloroacetic acid (5 : 1). The same procedure was then repeated for another two reaction mixtures changing the reaction time to 20 and 30 seconds respectively. For determining the initial concentration of hydrogen peroxide, the mixture of H_2SO_4 and trichloroacetic acid was added to the sample before homogenization. Each reaction mixture was then filtered. 4.0 ml of this filtrate were analyzed for hydrogen peroxide remaining by the addition of one drop of 1 per cent of ammonium molybdate and 2.0 ml of 10 per cent of KI and the liberated iodine was titrated with 0.005 N sodium thiosulfate (starch-iodine as indicator). The specific catalase activity of liver was expressed as K per g of dry weight where K was the mean of the observed first-order velocity constant at 10, 20 and 30 seconds respectively.

Manometric method used was essentially the same as used by Sizer (23). The

reaction was allowed to proceed in Warburg vessels and the amount of oxygen evolved from hydrogen peroxide was determined. Each vessel contained 2 ml of 0.01 M of phosphate buffer (pH 7.0) and 0.1 ml of 0.22 M of hydrogen peroxide, its final concentration being 0.01 M, and further, in the side arm, 0.1 ml of liver homogenate corresponding to 0.2 mg of wet liver tissue. After 4 minutes' shaking (120 strokes per minute) in an incubator at 38°C, the stop-cocks were closed and the reaction was started by tipping the homogenate into the buffered hydrogen peroxide solution. Readings were taken at intervals of 3, 4 and 5 minutes. Catalase activity was expressed as the amount of evolved oxygen (cmm) in 5 minutes per mg of dry weight of liver.

Results

Effect of Hypophysectomy.....The figures for the liver catalase activity of normal and tumor-bearing rats with and without hypophysis are summarized in Tables 1 and 2. It will be seen that in normal animals with intact hypophysis, the liver catalase activity in females is about 30 per cent lower than in males. In the case of tumor (fibrosarcoma)-bearing animals, the liver catalase activity is greatly reduced whether in males or females, values for K being decreased on an average from 61.4 to 31.3 in the case of males and from 39.8 to 21.3 in females (Table 1).

Table 1. Effect of Hypophysectomy on Liver Catalase Activity in Normal and Tumor-bearing Rats

Catalase activity was estimated by iodometry and expressed as K per g of dry weight of liver.

Animals		with Hypophysis		Surgical trauma	without Hypophysis	
		Normal	Tumor-bearing		Normal	Tumor-bearing
Male	1	58.4	30.5	46.0	97.8	117.0
	2	68.0	58.0	53.4	127.5	100.0
	3	59.8	31.8	73.0	113.0	93.5
	4	55.4	21.2	45.2	106.0	93.7
	5	83.5	27.4	71.0	72.5	62.8
	6	39.8	18.4	—	89.3	54.8
	7	64.6	31.9	—	71.8	125.0
Average		61.4	31.3	57.7	96.9	92.4
Female	1	36.8	23.9		81.5	57.8
	2	45.0	12.0		139.5	93.3
	3	24.0	10.6		116.5	—
	4	37.4	38.8		95.8	—
	5	55.8	—		—	—
Average		39.8	21.3		108.3	75.6

After hypophysectomy, however, the liver catalase activity rises from 61.4 to 96.9 in male rats and from 39.8 to 108.3 in female rats. It is also to be noted that the difference in the liver catalase level between both sexes appears to become negligible when the animals are hypophysectomized. In control animals traumatized by the same operative procedure as in hypophysectomy, but with hypophysis intact, there is no increase whatever in the liver catalase activity, as also shown in these Tables. Therefore, it is clear that the increase in liver catalase level observed in hypophysectomized rats is not an effect of the operation.

However, in the case of tumor-bearing rats from which the hypophysis had been removed prior to transplantation of the fibrosarcoma, the liver catalase activity did not show any marked decrease, as observed in the case of tumor-bearing animals with hypophysis intact. The value of K are only slightly decreased from 96.9 to 92.4 in males, and from 108.3 to 75.6 in females. It is true that in hypophysectomized rats, the growth rate of transplanted tumor is low as compared with that in normal with hypophysis. But it is to be noticed that the weight of tumor was progressively increased to 10 to 15 per cent of body weight of the host animal, in spite of the absence of the hypophysis.

It appears therefore that the liver catalase level once heightened by hypophysectomy is no longer affected by the presence of growing tumor. The results obtained by the

Table 2. Effect of Hypophysectomy on Liver Catalase Activity in Normal and Tumor-bearing Rats

Catalase activity was estimated with manometric method and expressed as the amount of oxygen (cmm) evolved in 5 minutes per mg of dry weiget of liver.

Animals		With Hypophysis		Surgical trauma	Without Hypophysis	
		Normal	Tumor-bearing		Normal	Tumor-bearing
Male	1	1590	1380	1000	2010	2170
	2	1620	1390	1040	2130	2280
	3	1650	970	1480	2210	1770
	4	1510	1080	1280	—	2050
	5	1650	1360	1350	—	1290
	6	1380	1090	—	1780	1420
	7	1290	1410	—	1480	1670
Average		1527	1240	1230	1922	1807
Female	1	1380	780		1810	1450
	2	1460	460		1730	1770
	3	1280	650		1470	—
	4	1180	1130		1590	—
	5	1260	—		—	—
Average		1312	755		1650	1610

Animal numbers in this Table correspond to those in Table 1.

manometric method are also exactly comparable with those obtained by the titrimetric (Table 2).

Effect of Hypophyseal Factors.....In order to examine the possible intervention of some hypophyseal factors in the control of liver catalase, a crude pituitary extract was prepared and administered to hypophysectomized rat. The results related to this subject are summarized in Tables 3 and 4.

Table 3. Effect of Hypophyseal Factor on Liver Catalase Activity in Normal and Hypophysectomized Rats

Catalase activity was estimated with iodometry and expressed as K per g of dry weight of liver.

Treatment		With Hypophysis		Without Hypophysis	
		Normal	Tumor-bearing	Normal	Tumor-bearing
Administration of Crude Pituitary;					
10 mg, 5 days	1	40.4		90.0	
	2	37.4		107.0	
	3	34.1		80.5	
	Average	37.3		92.5	
Administration of Crude Pituitary;					
25 mg, 5 days	1	20.7		37.7	
	2	49.5		43.1	
	Average	35.1		40.4	
Administration of Crude Pituitary;					
50 mg, 5 days	1	27.3		36.6	
	2	26.9		42.8	
	Average	27.1		39.7	
Administration of Growth Hormone:					
0.2 mg, 5 days	1	38.4		71.5	25.1
	2	28.2		58.8	24.6
	3	22.6		—	—
	Average	29.7		65.1	24.8
Administration of Growth Hormone;					
0.2 mg, 30 days	1	77.2		67.8	39.1
	2	80.5		33.7	61.7
	3	—		87.0	—
	4	—		95.0	—
	Average	78.9		70.5	50.4

When 25 mg or 50 mg of this crude pituitary powder were daily injected for 5 days into animals from which the hypophysis had been removed, it was found that the liver catalase activity was invariably lowered to almost normal level, showing no longer a high catalase level characteristic of hypophysectomized animals, although

the daily injections of 10 mg for 5 days were without effect. Also, the liver catalase activity in normal rats receiving the same dose of this extract was found to be considerably depressed. This was probably because excess of hypophyseal factors was not very favorable to the maintenance of the enzyme activity.

Next, an attempt was made to examine whether or not the pure pituitary growth hormone is also effective in decreasing the liver catalase activity in hypophysectomized rats. When 0.2 mg of growth hormone was daily injected into hypophysectomized rats for 5 days, the liver catalase activity was significantly decreased as compared with that in uninjected hypophysectomized rats (from the K value of 96.9 to 65.1).

Table 4. Effect of Hypophyseal Factor on Liver Catalase Activity in Normal and Hypophysectomized Rats

Catalase activity was estimated by manometric method and expressed as the amount of oxygen (cmm) evolved in 5 minutes per mg of dry weight of liver.

Treatment		With Hypophysis		Without Hypophysis	
		Normal	Tumor-bearing	Normal	Tumor-bearing
Administration of Crude Pituitary;					
10 mg, 5 days	1	1190		1830	
	2	990		1760	
	3	1030		1930	
	Average	1036		1840	
Administration of Crude Pituitary;					
25 mg, 5 days	1	680		1090	
	2	1120		1090	
	Average	900		1090	
Administration of Crude Pituitary;					
50 mg, 5 days	1	750		1150	
	2	960		1050	
	Average	855		1100	
Administration of Growth Hormone;					
0.2 mg, 5 days	1	1365		1470	1140
	2	1170		1965	790
	3	1250		—	—
	Average	1261		1718	965
Administration of Growth Hormone;					
0.2 mg, 30 days	1	1700		1760	1440
	2	1490		1190	1800
	3	—		2260	—
	4	—		1790	—
	Average	1595		1750	1620

Animal numbers in this Table correspond to those in Table 3.

In the case of tumor-bearing animals without hypophysis, the effect of the injection of growth hormone was far greater than in normal animals from which the hypophysis had been removed, K value for hypophysectomized-tumor-bearing control being 92.4 on an average and that for experimentals receiving growth hormone being 24.8.

Therefore, two conclusions may be drawn from the results hitherto obtained. First, the depression of liver catalase activity, which is one of the most evident characteristics of the tumor-bearing animals, is no longer observed when animals are deprived of hypophysis prior to the inoculation of tumor, although the tumor increases progressively in weight even in the absence of the pituitary. Secondly, catalase activity of liver once made heightened by hypophysectomy is invariably decreased to almost normal level by the administration of either the crude pituitary extract or growth hormone, whether in normal or tumor-bearing animals. indicating that some hypophyseal factors, particularly the growth hormone, are involved in the control of liver catalase.

Lastly it is to be noted that in the case of normal rats with hypophysis intact, growth hormone always caused depression of liver catalase level as observed in experiments with crude pituitary powder. Furthermore, it was found that when growth hormone was administered for prolonged period in hypophysectomized rats (daily dose of 0.2 mg for 30 days), the liver catalase was also reduced to normal (from 96.9 to 70.5), although in normal rats with hypophysis, the injection of growth hormone for prolonged period resulted in an increase of the enzyme level, probably due to the acceleration of growth by the continuous administration of growth hormone. That liver catalase activity parallels the growth of the animal has been reported by Seabra and Deutsch (24).

Discussion

The fact that liver catalase level is higher in male animals than in female has been reported by Hargreaves and Deutsch (5) and Adams (13, 14). This difference in liver catalase activity between the sexes may be attributable to the deficiency of testosterone in the female, since the removal of testis was reported to cause depression of liver catalase in mice (13, 14, 15). In rats also, similar results were obtained in an unpublished experiment recently carried out by the author. On the other hand, adrenalectomy was also reported to be effective in decreasing the liver catalase level in rats (16) and in mice (13, 14, 15).

In the present study, it was shown that hypophysectomy always results in an increase of liver catalase activity in rats and that under such condition no difference in catalase level is observed between the sexes. These results do not necessarily seem to be consistent with the above mentioned conception that testosterone is responsible for the high level of catalase in male animals, for hypophysectomy will cause depres-

sion of gonadotrophin and lead to the deficiency of androgens. However, in view of the fact that adrenalectomy causes depression of liver catalase, it is possible that the hypophyseal hormone(s) is acting through the intervention of adrenal hormones such as ACTH.

The main point of importance in the present paper is that the depression of liver catalase activity is no longer observed in tumor-bearing rats after hypophysectomy and that the injection of growth hormone is very effective in decreasing the liver catalase in these animals, although the hormone exerts a similar depressing effect on liver catalase in normal animals. Gaebler and Mathies (17) also reported that growth hormone could depress the liver catalase in hypophysectomized female rats, though they did not perform experiments on tumor-bearing animals.

Summary

1. The influence of hypophyseal factors on liver catalase level of tumor-bearing animals was examined, using male and female albino rats bearing fibrosarcoma as material. Catalase activity was measured by the two methods; the iodometric titration and the manometric method.

2. Although the liver catalase activity was two to three times as high in the hypophysectomized rats as in normal controls, it was found to be reduced to normal level by the intraperitoneal administrations of 25 or 50 mg of beef anterior pituitary (as crude acetone powder in saline suspension). A similar result was also obtained with the daily dose of 0.2mg of pure growth hormone for 5 days.

3. The liver catalase activity in hypophysectomized animals was not decreased at all in spite of the presence of a growing tumor. However, the depression of liver catalase activity was invariably observed in hypophysectomized-tumor-bearing animals when growth hormone was administered (daily dose of 0.2 mg for 5 days).

4. The present data suggest that the catalase depressing factor produced by tumor may act through some of the hypophyseal factors and that growth hormone plays an important role in the regulation of liver catalase level, whether in normal or tumor-bearing animals.

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EFFECT OF HYPOPHYSECTOMY ON LIVER CATALASE ACTIVITY IN TUMOR-BEARING MICE*

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The reduction of liver catalase activity has been shown as one of the characteristics of the biochemical pattern in tumor-bearing animals (Greenstein (1)). However, the alteration of liver catalase level also occur as a result of hormonal imbalance. A series of experiments concerning the relationship between hormonal factors and mouse liver catalase has been reported by Adams (2, 3, 4). It appears from his data that mouse liver catalase is strongly affected by the removal of testis or of adrenals, the normal level being again restored by the injection of testosterone or cortisone. However, he did not perform experiments on the effect of hypophyseal factors on mouse liver catalase.

In the previous paper (5), the author has reported that liver catalase activity of hypophysectomized rat is considerably increased and that in hypophysectomized tumor-bearing rats this heightened level of catalase is no longer affected in spite of the presence of growing tumor. It was also suggested that growth hormone plays an important part in the regulation of liver catalase level, whether in normal or tumor-bearing animals.

In the present study, similar experiments were carried out using mice as material and the results indicated that the effect of tumor on the host liver catalase was detectable only when the hypophysis of the host was kept intact.

Materials and Methods

Hybrid albino male mice weighing from 17 to 25 g were used. Hypophysectomy was perfomed through the parapharyngeal approach under ether anesthesia with the modified methods of Selye (6) and Lestroh and Jordan (7). Hypophysectomized mice were used for experiments after a cessation of body weight increase has been confirmed 2 weeks after operation. Completeness of hypophysectomy was confirmed at autopsy by examination of the atrophy of the testis. Occasionally a histological observation on the testis and adrenal was also done. Ehrlich ascites tumor was used as a transplantable tumor, transplantation into hypophysectomized mice being performed 3 weeks after operation.

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Catalase activity was estimated by the iodometric titration, hydrogen peroxide remaining after known time intervals being determined. Also the manometric method in which oxygen generated by the decomposition of hydrogen peroxide was estimated, was adopted. The former is essentially based on the method of Herbert (8) and the latter on that of Sizer (9). Details have already been described in the previous paper (5).

Results

The effect of hypophysectomy on liver catalase level was examined in both normal and tumor-bearing mice and the results are given in Tables 1 and 2. It will be seen

Table 1. Effect of Hypophysectomy on Liver Catalase Activity in Normal and Tumor-bearing Mice

Catalase activity was measured by iodometry and expressed as K per g of dry weight of liver.

Animals		With Hypophysis	With Incomplete Hypophysis	Without Hypophysis
Normal	1	39.1	71.0	79.2
	2	72.5	44.2	52.2
	3	64.0	50.0	53.7
	4	63.2	39.8	45.8
	5	57.4	49.8	37.2
	6	30.7	64.9	—
	7	—	43.7	—
	Average	54.5	51.9	53.4
Tumor-bearing	1	22.0	29.9	44.6
	2	25.0	40.0	42.5
	3	28.8	38.3	62.3
	4	22.3	34.4	64.0
	5	18.7	23.2	38.9
	6	14.7	27.1	—
	7	30.3	26.1	—
	8	28.2	51.8	—
	9	—	15.3	—
	10	—	16.9	—
	11	—	18.8	—
	12	—	34.2	—
	13	—	31.2	—
	Average	23.8	29.7	50.5

that liver catalase activity is little affected in the case of normal mice by hypophysectomy or incomplete hypophysectomy in which about one half of the hypophysis has been kept intact. The liver catalase level of hypophysectomized mice is 53.4 on an average, those of normal and incompletely hypophysectomized mice being respectively

54.5 and 51.9. It does not seem therefore that the hypophyseal factors are involved in the regulation of liver catalase in the case of normal mice. This result is somewhat surprising, since in the case of rats hypophysectomy always leads to the increase of liver catalase level as shown in the previous paper (5).

However, when Ehrlich ascites tumor is transplanted into mice from which the hypophysis has been completely removed, the depression of liver catalase no longer occurs, indicating that for the tumor to exert its influence on the liver catalase of the host, some of the hypophyseal factors is essential as in the case of tumor-bearing rats. That liver catalase activity is evidently reduced following the transplantation of the tumor into normal mice with intact hypophysis is also clearly seen in Tables 1 and 2. In the case of incomplete hypophysectomy also, the depression of catalase due to tumor is obvious. However, it is to be noted that also in the case of mice, the transplanted tumors carry on growth, though at a very slow rate.

Table 2. Effect of Hysophysectomy on Liver Catalase Activity in Normal and Tumor-bearing Mice

Catalase activity was estimated by the manometric method expressed as the amount of oxygen (cmm) evolved in 5 minutes per mg of dry weight of liver.

Animals		With Hypophysis	With Incomplete Hypophysis	Without Hypophysis
Normal	1	1350	1540	1570
	2	1720	1110	1085
	3	1390	1045	1220
	4	1085	1000	1300
	5	1530	1890	1490
	6	1140	—	—
	7	—	1690	—
	Average	1353	1379	1333
Tumor-bearing	1	935	963	1025
	2	790	—	1430
	3	795	870	1280
	4	760	1370	1570
	5	850	900	1220
	6	1030	1080	—
	7	873	850	—
	8	1040	1270	—
	9	—	1010	—
	10	—	940	—
	11	—	1270	—
	12	—	1490	—
	13	—	1360	—
	Average	876	1029	1305

The animal numbers in this Table correspond to those of Table 1.

Exactly the same conclusion was reached for experimental series using the manometric method.

Discussion

The purpose of this study was to examine the possible role of hypophysis in the control of liver catalase level in normal and tumor-bearing mice. As regards the part played by the hypophyseal target organs such as the testis and adrenal on mouse liver catalase, a considerable attention has been directed by Adams (2, 3, 4). He demonstrated that mouse liver catalase was markedly reduced by the removal of testis and of adrenals, and that the reduction could be restored to normal by the administration of testosterone and cortisone respectively. However, a complete restoration of catalase level could not be obtained by the compensatory administration of testosterone to adrenalectomized animals or that of cortisone to castrated animals. It was suggested therefore that testicular or adrenal hormone is independently involved in the regulation of liver catalase level.

In the present study, it has been shown that the removal of hypophysis does not affect the level of liver catalase in normal mice, although under such conditions there should be no production of testosterone or cortisone. If it is correct that a single hormone such as testosterone or cortisone is essential for the control of liver catalase, hypophysectomy is expected to produce a marked effect on the catalase level. But this is not the case at least under the conditions of the present experiment. Therefore it would appear that at least in mice, liver catalase level is regulated by the intervention of various hypophyseal hormones secreted from the respective target organs.

However, as regards the effect of hypophysectomy on liver catalase in tumor-bearing rats, the results obtained in the present study show fairly conclusively that hypophysis is essential for the causation of the depression of liver catalase activity in these animals. This result is in accord with that in the case of tumor-bearing rats (5) and strongly suggests that some hypophyseal hormone such as growth hormone is involved in this process.

Summary

1. An attempt was made to examine whether or not some hypophyseal factors is involved in the control of liver catalase level in tumor-bearing mice, male hybrid mice being used, and hypophysectomy performed with parapharyngeal approach. Catalase activity was estimated by the two methods; iodometry and manometric method. As a transplantable tumor, Ehrlich acsites tumor was used.

2. Hypophysectomy does not increase the liver catalase level in the case of normal mice as opposed to the case of rats. In the hypophysectomized-tumor-bearing mice,

however, there was found no increase in liver catalase level in spite of the presence of growing tumor, although in the case of mice with hypophysis intact or with incomplete hypophysis, there was a considerable reduction of liver catalase level as a result of tumor transplantation.

3. The result suggests that hypophysis is indispensable for the reduction of liver catalase in tumor-bearing animals.

Aknowledgment

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EFFECT OF X-RADIATION ON THE ASCITES TUMORS: DIFFERENCE IN RADIOSENSITIVITY AMONG VARIOUS TRANSPLANT-STRAINS OF THE ASCITES HEPATOMA OF THE RAT*

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INTRODUCTION

The ascites hepatoma is produced by procedures of ascitic conversion of the aminoazodye-induced hepatoma of rats. Twenty-five different strains of the tumor have been established by serial transplantations and kept for comparative studies in this laboratory. The primary tumors of the 25 transplant-strains of the ascites hepatoma originated each in separate animals. They are naturally all common in the normal ancestral cell, the liver cell. However, the comparative studies of their general characteristics, such as growth velocity, transplantability, ascitic picture, and chromosome number of tumor cll, demonstrated, against expectations based on common knowledge of the tumor, that they never did agree with each other (18, 19). That the ascites hepatoma is reproducible and shows individual variaties is the point which distinguishes the ascites hepatoma from any other kind of ascites tumor available, which is not provided with its duplicates to be compared with each other.

Among individual differences of the ascites hepatoma, the most remarkable was that they varied in the response to anti-cancerous treatment. It was first observed in the susceptibility to HN_2N-oxide (nitromin) by Satoh (16). As to compounds other than nitromin, which also belong to the alkylating agents, such as TEM, myleran, and thio-TEPA, similar strain-differences were already demonstrated (20). Of further interest is whether this kind of strain-differencse of the ascites hepatoma extends to the susceptibility to radiations. The present paper deals with the effect of X-radiation upon various ascites hepatomas and the difference in their radiosensitivity.

MATERIALS AND METHODS

Tumors: Twenty transplant-strains of the ascites hepatoma, i. e., strains AH 130,

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**Directed by Prof. Tomizo Yoshida, Director of the Medical Institute of Sasaki Foundation and Department of Pathology, Faculty of Medicine, University of Tokyo.

7974, 601, 602, 66, 63, 149, 39, 49, 99, 66F, 322, 414, 21, 423, 364, 318, 13, 62, and 408, were used in this study.

Animals: Eight hundred and fifty-five rats (Japanese common albino, of which 2/3 were males), weighing 80 to 110 grams in body weight, were used. The diet was composed of crushed wheat and vegetables, which were given *ad libitum* with water.

Transplantations and Radiations: The age of tumor ascites used as inoculum was, as a rule, settled at half of the median survival period of the animals of each tumor strain. The tumor ascites, 5 ml, of each strain was taken from more than 2 animals which were confirmed to have tumor ascites showing the pure culture state of tumor cells, and was mixed with 0.03 mg of heparin, and then diluted with physiological saline solution so that the diluted ascites contained 10^8 tumor cells per ml. The diluted ascites was aspirated into 3 glass syringes of 1 ml, and 0.1 ml of the content of each syringe was injected intraperitoneally into 2 animals. Six animals in total, thus inoculated with 10^7 tumor cells, of each strain served as control.

Immediately after this transplantation, the remaining ascites in the 3 syringes was exposed to X-ray. When irradiated with 200r, the shutter of the X-ray tube cover was closed and 0.1 ml of the irradiated tumor ascites in each syringe was injected to the abdominal cavity of one animal, namely 3 animals in total. The shutter was reopened afterwards, the remaining tumor ascites was irradiated with additional 800r, so that the total dose of X-ray was 1,000r, and it was transplanted intraperitoneally to 3 animals. The does of inoculum was 0.1ml in each case. In the same way, 0.1 ml of the remaining tumor ascites was inoculated into 3 animals after the exposure of 2,000r, 3,000r, 4,000r, and 5,000r in total.

At the time of radiation, the syringes were put on a disk of acrylic resins kept at the height of 90cm above the floor, and a supporting plate of acrylic resins was inserted between the disk and the X-ray tube cover in order to keep the focus-syringe distance constant (Fig. 1). X-ray was generated at 200 kVp and 20 mA, filtered by 0.7 mm Cu and 0.5 mm Al; HVL was 1.5 mm Cu. The focus-syringe distance was 21.9 cm, and the dose rate 286r/min. Doses were measured by using as a phantom a 5ml syringe, the cylinder of which was the same in thickness of wall as that of the described syringes. The fluctuation of X-ray doses was

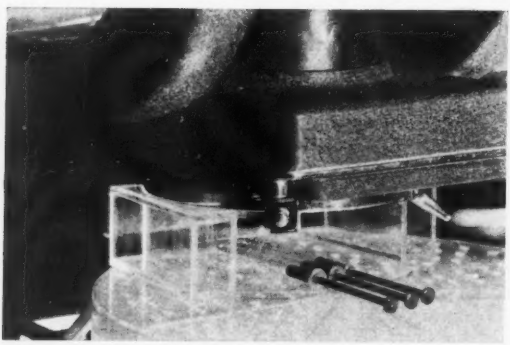

Fig. 1. Photograph showing the arrangement to keep the focus-syringe distance constant at the time of radiaton.

within ±5 per cent.

Procedures of Examination: A drop of ascitic fluid was taken from every animal 1, 2, 3, 4, 5, 10, 15 and 20 days after transplantation with irradiated tumor cells respectively, and smear preparations were stained with Wright's and Giemsa's solutions. The number of tumor cells as well as their cytological features were examined. The animals survived more than 20 days were occasionally examined thereafter, until the 60th day when all the animals were sacrificed for the examination of tumors.

Thus, in the case that the inoculated 10^7 tumor cells disappeared completely from the ascitic fluid of the animals, and that the growth of tumor cells could not be detected until 60 days after inoculation, all the tumor cells were regarded to have received the "lethal effect" and the minimum X-ray dose necessary to cause the effect was demonstrated as the "lethal X-ray dose" in each tumor strain.

The animals died of accidental causes or confirmed to be naturally insusceptible to tumors were omitted, but in the animals in which the irradiated cells grew temporarily and disappeared later the lethal effect was decided as "negative".

As to 3 strains of the ascites hepatoma, AH 149, 130 and 7974, comparison was made on the life span of the tumor animals transplanted with 10^7 irradiated tumor cells. In these cases, the inocula were given 2,000r or 4,000r *in vitro* before the inoculation. The ascitic fluid of rats was also examined 2, 10, 20, 30, 40, 50 and 60 days after transplantation, and all the surviving rats were killed at the time of the last examination.

Results

A. Lethal X-ray Doses for Various Strains of the Ascites Hepatoma.

Table 1 shows the results in transplantation of the irradiated tumor cells *in vitro* with various amounts of X-ray. The animals in which the tumor did not grow are regarded as those which received the tumor cells irradiated lethalically. Thus for example, in case of AH 39, where the inoculum irradiated with 2,000r did not grow in all receipients, this dose of 2,000r was regarded as the lethal dose of AH 39.

Based on these results, the lethal X-ray dose in each tumor strain is illustrated clearly in Chart 1. As shown in the chart, 4 strains of the ascites hepatoma were most resistant to X-radiation. They are strains AH 99, 13, 66F, and 7974, and the dose of X-ray to kill them completely is, indeed, 5,000r. On the other hand, three ascites hepatomas, i. e., AH 39, 149, and 601, belong to the most sensitive group to radiation among the whole tumor tested. They were killed completely by 2,000r. Striking is the fact that the lethal X-ray doses of these 2 groups differ from each other by about 3,000r. The remaining 13 strains of the ascites hepatoma are inbetween of the said resistant and sentitive groups.

Table 1. Results in growth of tumor grafts (10^7 cells) irradiated *in vitro* with various amounts of X-ray.

Strains		Control	200r	1,000r	2,000r	3,000r	4,000r	5,000r
AH	39	12/12	6/6	1/3	0/5	0/5	0/5	0/4
	149	18/18	9/9	4/8	0/7	0/7	0/8	0/8
	601	6/6	3/3	3/3	0/2	0/2	0/1	0/1
	423	12/12	6/6	4/5	1/4	0/3	0/1	0/3
	66	6/6	3/3	3/3	1/2	0/3	0/3	0/3
	49	6/6	3/3	3/3	1/2	0/2	0/3	0/3
	414	12/12	6/6	4/5	2/5	0/3	0/4	0/5
	130	18/18	9/9	7/8	5/8	0/4	0/8	0/6
	62	6/6	3/3	2/2	2/3	0/1	0/2	0/2
	63	6/6	3/3	2/2	1/1	0/3	0/3	0/2
	322	6/6	3/3	2/2	3/3	0/3	0/2	0/3
	602	12/12	6/6	6/6	5/5	0/3	0/2	0/3
	364	12/12	6/6	6/6	5/6	1/5	0/4	0/4
	408	6/6	3/3	3/3	2/2	1/3	0/2	0/1
	21	6/6	3/3	2/2	2/3	1/2	0/2	0/1
	318	12/12	6/6	6/6	4/6	4/5	0/2	0/6
	99	12/12	6/6	6/6	4/4	1/4	1/5	0/5
	13	11/11	6/6	6/6	5/5	3/5	1/6	0/6
	66F	6/6	3/3	3/3	2/2	1/1	1/3	0/2
	7974	17/17	9/9	9/9	9/9	7/9	5/9	0/6

Denominators represent the number of valid recipients of the irradiated grafts, while numerators represent the number of the animals in which the tumors did grow.

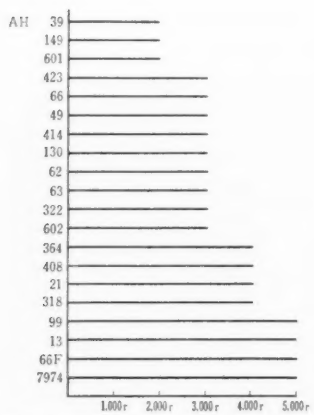

Chart 1. Lethal X-ray dose for 10^7 cells of each strain of the ascites hepatoma.

B. Growth and Cytological Changes of Irradiated Tumor Cells after Transplantation.

Chart 2A shows the grade and continuation of tumor cell reduction, degeneration of cells, abnormal mitosis, abnormal interphase and increase in cell volume, observed through 1 to 5 days after inoculation of tumor cells exposed to single radiation of 1,000 to 5,000r. These changes occupy the majority of cytologically observed X-radiation effects on tumor cells, as described in other paper (11). In the figure each square for each item was horizontally as well as vertically divided into 5 equal parts, so as to be divided into 25 equal small squares. The horizontal axis

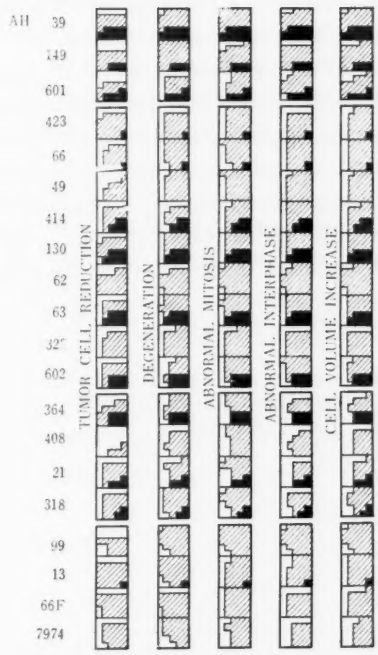

Chart 2A Various morphological changes of ascites hepatoma cells caused by doses of 1,000r–5,000r.

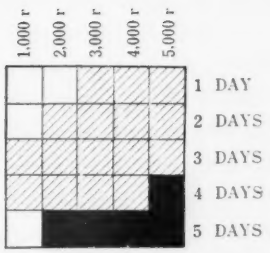

Chart 2B Direction for reading of squares in chart 2A. Square of "abnormal interphase" of AH 601.

was lined with X-ray dose of 1,000r, 2,000r, 3,000r, 4,000r, and 5,000r from the left to the right, and the vertical axis was lined with the time of 1, 2, 3, 4, and 5 days after radiation from the top to the bottom. When the marked cellular changes caused by radiation were observed in more than half of all the tumor cells, oblique lines were drawn in the small square corresponding to the described dose and time. When no tumor cells were detected at all in the smear examined, the small square was blackened. Thus for example, the square of "abnormal interphase" of AH 601 should be read as follows (Chart 2B): No distinct changes of tumor cells could be observed 1 to 2 days after radiation with 1,000r, while abnormal figures of interphase cells occupied more than half of the whole interphase tumor cells through 3 to 4 days after radiation; but the cells ofsuch abnormality in interphase could not be detected at all 5 days after radiation, because of the reproduction of tumor cells. In the case of radiation with 2,000r, it is shown that the changes began to appear 2 days after radiation and the tumor cells disappeared 5 days after.

The time of disappearance of the inoculated tumor cells which were irradiated before the inoculation was different by each tumor strain. In some strains, e. g. strains AH 39, 130, 63, 602, and 364, etc., tumor cells disappeared completely 4 days after radiation with 3,000r; while, in other strains, e. g. strains AH 49, 62, 322, 408, 99, 66F, and 7974, etc., tumor cells still remained even 5 days after radiation with 5,000r. However, even in the strains such as AH 39 and 130 in which tumor cells decreased early, the cells did not disappear for 3 days after radiation with 5,000r,

and, morever in the strains AH 149, 66, 62, and 99, tumor cells could be found for 3 days even after the exposure to 20,000r. From these facts, it may be considered that a rapid cytolysis could not be caused only by a large dose of X-ray, and that it would take at least 4 days for all of the irradiated ascites hepatoma cells to die.

As a whole, the most remarkable cellular changes appeared in strains AH 39 and 149; while the slightest changes appeared in strains AH 408 and 7974. Tha tthe changes were so slight in AH 408 was, however, because of the fact that the changes took place slowly; on the other hand, the changes in AH 7974 were relatively early and intensive, but, on account of the active reproduction of tumor cells, the frequency of those changed cells was low among the whole tumor cells examined.

C. Survival Periods of Ascites Hepatoma Animals Transplanted with the Tumor Cells Exposed to Single X-Radiation *in vitro*.

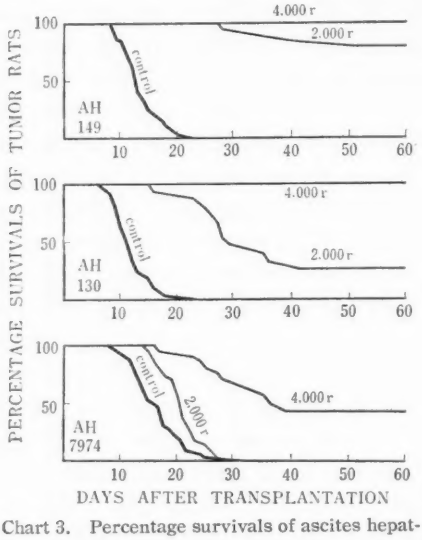

Chart 3. Percentage survivals of ascites hepatoma animals after the intraperitoneal transplantation of 10^7 tumor cells treated with single X-radiation *in vitro* with 2,000r or 4,000r.

Percentage survival curves of the tumor rats which were inoculated intraperitoneally with 10^7 ascites hepatoma cells exposed *in vitro* to single radiation with 2,000r or 4,000r were indicated in Chart 3. The strains AH 149, 130, and 7974, were used as the materials for this examination, because the survival periods of the rats bearing these tumors was similar but the lethal X-ray dose of those tumors was different as shown already in Chart 1. As exhibited in Chart 3, the prolongation of life of tumor animals and/or the percentage of rats which were completely free from the tumor (the rats in which the tumor cells showed "lethal effect" of X-ray) were high in AH 149 and low in AH 7974. The strain AH 130 was inbetween in these aspects.

Discussion

A comparison of radiosensitivity was made between the mammary tumors autogenous to the dba and C3H mice by Goldfeder, who found that the C3H tumor growing *in vitro* was more radioresistant than the dbrB tumor growing *in vitro* (6); but that the X-ray dose, which was required to prevent the tumor implant from growing in the same strain of mice, was smaller in the C3H tumor than in the dbrB tumor (5);

and that the X-ray dose, which was required to cause the total regression of well-developed solid tumors by means of X-radiation *in vivo*, was also smaller in the C3H tumor (8). Moreover, from the results that the oxygen uptake, aerobic glycolysis and growth rate of the dbrB tumor were nearly 3 times as much as those of the C3H tumor (7, 8), she stated that the slower growing tumor was more radiosensitive than the faster growing tumor *in vivo* (8). However, she reported later that the C3HB tumor, which was more anaplastic and contained less stroma, was more radioresistant than the DBAH tumor, which contained more stroma and consisted of uniform tumor cells (9). On the other hand, Cathie who investigated some 5,500 sections of human tumors reported that "it will be impossible to give a certain forecast of tumor sensitivity from the usual histologic preparations" (3). Some morphological studies were made on chromosome number and radiosensitivity (4, 10), but the results were not in agreement. A cytochemical research on the 3 lines of the Ehrlich ascites tumor, which were different in the number of chromosomes, was presented by Caspersson (2). He found that the difference of radiosensitivity could hardly be demonstrated among those tumors.

The present study using 20 different ascites hepatomas demonstrated that even the tumors derived from one and the same ancestral cell, the liver cell, varied in the X-ray sensitivity so far examined. Considerations on various aspects related to the radiosensitivity of tumors are as follows.

Lethal X-ray dose and biological as well as morphological characteristics of tumors.

General biological and morphological characteristics of each strain of the ascites hepatoma used were shown in Table 2. These are the rate of transplantation, the growth velocity*, the grade of polymorphism of tumor cells, the chromosome number of tumor cells, the concentration of tumor cells in the ascitic fluid, the hemorrhagic tendency and the histological types of tumors.

As clearly accepted from Table 2, it is beyond question that there is a very close

*The cells of the ascites hepatoma proliferate not only in the ascitic fluid but in various tissues. The tumor cells conglomerate and form "hepatoma islands" in ascitic fluid, while they form solid nodules in the tissues where they infiltrate. Therefore, it is difficult to show numerically the state of multiplication of tumor cells in the strict sense. In this connection, the author examined the growth velocity by the following method: By utilizing 6 animals in each tumor strain, the avarage survival periods of the animals which died of the tumor after inoculation of 10^7 tumor cells was calculated, and also the grade of proliferation of tumor cells was checked at the time of animals' death in regard to the 9 parts such as the abdominal cavity, the thoracal cavity, the abdominal wall, the peritoneal surface, the greater omentum, the fatty tissues around the genital organs, the lymphonodes in the abdominal cavity, the lymphnodes of mediastinum, and the lymphnodes of other regions. The grade of tumor cell proliferation was divided into 4; when no tumors could be observed, it was grade O; when slight tumors were noticed, it was grade 1; when moderate, it was grade 2; when very →

relation between the growth velocity and radiosensitivity of tumors. The growth indeces of 4 strains of the ascites hepatoma, i. e. strains AH 66F, 99, 13, and 7974,

Table 2. Lethal X-ray doses and various biological natures of the ascites hepatoma.

Strains of the ascites hepatoma	Lethal X-ray dose	Transplantation rate	Growth velocity (growth index)	Grade of polymorphism	Mode of chromosome number	Tumor cell concentration	Hemorrhagic tendency	Histological type*
AH 39	2,000r	80%	3.0	++	49-50***	high	−	II
149	2,000r	88%	6.4	++		low	+	IV
601	2,000r	82%	3.6	+	68	low	−	III
423	3,000r	51%	3.3	+		high	+	
66	3,000r	90%	9.2	+	70	high	+	II
49	3,000r	76%	6.8	+		high	+	IV
414	3,000r	82%	3.5	+		high	+	
130	3,000r	97%	8.5	+	43	high	+	I
62	3,000r	84%	5.0	+		high	+	
63	3,000r	80%	4.1	++		low	−	
322	3,000r	60%	8.5	+		high	++	III
602	3,000r	92%	5.4	+	68	low	−	III
364	4,000r	93%	10.0	+		high	++	
408	4,000r	86%**	8.6	+		low	++	
21	4,000r	77%	10.0	++		low	+	II
318	4,000r	77%	8.8	+		high	++	IV
99	5,000r	91%	10.8	++		low	++	
13	5,000r	94%	10.0	+	38***	high	+	I
66F	5,000r	91%	17.0	+		high	+	I
7974	5,000r	96%	10.0	+	49	high	+	III

* Histological types of intrahepatic tumor growth (13). Type I: Solid nests with no characteristic arrangemnet of tumor cells, accompanied by leukemia-like infiltration into sinusoids. Type II: Expansive solid nests with no characteristic arrangement of tumor cells. Neither leukemia-like infiltration nor cystic formation. Type III: Expansive solid nests composed of various sized conglomerates of tumor cells, partially showing cystoadenomatous picture. Type IV: Predominantly cystoadenomatous.

** Unpublished data (15).

*** Unpublished data (14).

→ Intensive, grade 3.

Thus, the index of growth velocity (growth index) of each strain was determined with considerations of the above two results. Take an example, the rats of strain AH 130 died various days after the inoculation, but their survival periods was 13 days on an average. The grade of tumor cell proliferation of the animals concerning the above-said 9 parts were, on an average, 3, 0, 2, 1, 2, 1, 2, 0, and 0, so that the sum was 11. This value 11 means the growth index of ascites hepatoma AH 130 at 13 days after transplantation. However, the growth index 8.5 at 10 days after transplantation which was caluculated by means of proportion from the above value 11, was shown in Table 2. The indices of all other tumor strains in the table were also those at 10 days after transplantation. The growth index appears to show characteristics of growth of each tumor strain pretty exactly and presents a convinient method of analysis for comparative studies of ascites tumor growths. It is understood that the tumor which shows the small index value grows slowly and *vice versa*.

which show the largest lethal X-ray dose 5,000r, are 17.0, 10.8, 10.0, and 10.0 respectively and belongs to the largest growth indices. The average of growth indices of these 4 tumors is 12.0, while other groups of ascites hepatomas, the lethal dose of them being 2,000r, 3,000r, or 4,000r, show the average growth indeces 4.3, 6.0, or 9.4 respectively. This means that the slower growing tumor is more radiosensitive than the faster growing tumor and *vice versa.*

The susceptibility of tumors to anti-cancer compounds, especially, the "lethal nitromin dose" **(17) of each strain of the ascites hepatoma was shown in Table 3 and compared with the lethal X-ray dose of the tumor. The strain AH 7974 is considerably resistant to both X-ray and nitromin and AH 130 belongs to the tumors which are sensitive to both of them. However, AH 149, the most resistant tumor to

Table 3. Lethal X-ray doses and lethal nitromin doses for the ascites hepatoma.

Strains of the ascites hepatoma	Lethal X-ray dose (10^7 cells)	Lethal nitromin dose* (10^8 cells)
AH 39	2,000r	10γ
149	2,000r	1,000γ
601	2,000r	>5γ
423	3,000r	>100γ
66	3,000r	500γ
49	3,000r	>10γ
414	3,000r	10γ
130	3,000r	5γ
62	3,000r	100γ
63	3,000r	100γ
322	3,000r	>100γ
602	3,000r	
364	4,000r	>100γ
408	4,000r	>20γ
21	4,000r	>100γ
318	4,000r	>50γ
99	5,000r	>5γ
13	5,000r	5γ
66F	5,000r	>10γ
7974	5,000r	100γ

* Unpublished data (17).

**Dr. H. Satoh examined the "lethal nitromin dose" of the ascites hepatoma by the following procedure: To 1ml. tumor ascites tapped from 4-day-old tumor animal——containing 100–200 million tumor cells——is added in the test tube various doses of nitromin (1, 5, 10, 50, 100, 500, or 1,000γ) dissolved in 1ml saline solution. After 30min. incubation at 37°C, the whole content of the tube, 2ml, is inoculated into the peritoneal cavity of normal rats. When the amount of given substance be sufficient, the cells are prevented from their proliferation thereafter. The procedure is repeated, and the minimum amount of nitromin required to kill all of the tumor cells (lethal nitromin dose) is determined.

nitromin, is one of those strains which are most sensitive to X-ray. Therefore, it may be said that no significant correlations can be found between the X-ray and the nitromin sensitivity of ascites hepatomas.

As to other characteristics of the ascites hepatoma, such as the transplantation rate, ascitic pictures, chromosome numbers of tumor cells, histological types, etc., it is quite impossible to find out any significant correlations with the radiosensitivity so far examined.

Lethal X-ray doses and the grade of cytological changes of irradiated tumor cells.

As already demonstrated in Chart 2A, the grade of changes of irradiated tumor cells was different in each tumor strain. However, the tumors in which the changes such as tumor cell degeneration, abnormal mitosis of tumor cells, abnormal figures of interphase cells, and increase of tumor cell volume, appeared more considerably, were strains AH 39, 149, and 99. These tumors are provided in their ascitic pictures with a common characteristic that the polymorphism of tumor cells is so remarkable (Table 2). Therefore, it may be said generally that the cytological changes of irradiated tumor cells occur more considerably in the tumor whose cells show marked polymorphism. However, it is not always the radiosensitive tumor strains that show such remarkable changes. On the other hand, strains AH 49 and 322, just like the strains AH 408 and 7974, presented far less cytological changes. This might be explained that the lethal X-ray dose is related closely to the growth velocity of the tumors, and the grade of cytological changes of tumors has a close relation to the polymorphism of tumor cells, and yet there is no relationship found between the growth velocity and the cellular polymorphism of tumors.

Examination on various aspects related to the radiosensitivity of tumors revealed that the sensitivity had a close relation to the growth velocity of tumors, and that a slower glowing tumor was more sensitive to radiation than a faster growing tumor as described already. However, there are still some points to be discussed as follows:

The reason why the ascites tumors were used instead of solid tumors:

There might be some opinions that solid tumors should be used in this kind of study because most of human tumors are usually in solid form. However, it is quite difficult to analyse the phenomena quantitatively when we use solid tumors. Thus for example, the accurate number of tumor cells can not be obtained in the use of solid tumors because of intermingling stroma tissues, such as blood vessels. If we use solid tumors for local radiation instead of ascites tumors for *in vitro* radiation, there might be several other factors to be considered to analyse the differential sensitivity of the tumors. There are different characteristics in either invasiveness into the tissues or transmission into the circulating blood among these strains of the ascites hepatoma (20). Based on these considerations, the use of ascites tumors seems to be adequate to carry out this kind of study to demonstrate the radiosensitivity

difference among many strains of tumors.

On the use of the lethal X-ray dose as a criterion for radiosensitivity:

There might be several ways to analyse the radiosensitivity of tumors, such as the mitotic rate of tumor cells and cytological changes of the irradiated tumor cells. The ascites hepatoma is composed of conglomerated hepatoma cells (hepatoma islands) as well as isolated tumor cells in the ascitic fluid. Since there is a considerable difference in the rate of mitotic cells of the islands belonging to one and the same strain of the tumor it is almost impossible to examine the tumor-strain difference in radiosensitivity by means of the mitotic rate. On the other hand, the grade of cytological changes of irradiated tumor cells is not taken as a clearcut criterion for this comparative study. Moreover, there is a fact that, as stated already, those cytological changes could be found remarkably in the limited tumors with characteristic of cellular polymorphism in ascites. Therefore, examinations of the lethal X-ray dose, the minimum X-ray dose necessary to kill all of the definite number of tumor cells of each tumor strain, was performed as the only, simple and reliable way to know the difference on radiation effects of various tumor strains.

Concerning Bergonié-Tribondeau's law:

Finally, Bergonié-Tribondeau's law (1) is to be discussed from the results of this study. Bergonié-Tribondeau's law——"the stronger effect of radiation is exerted on the least differentiated cells and on those of greatest reproductive ability"——appears to be based on the judgement that the faster growing tumor is more radiosensitive than the slower growing tumor. The judgement would come, on the one hand, from the fact that changes induced by radiations occurred earlier in the faster growing tumor than in the slower growing tumor, and, on the other hand, from the fact that the former was reduced in size earlier and more remarkably than the latter after radiation in both clinical and experimental observations. However, attention should be paid to the fact that the tumors they used were not the ascites tumors, but the solid tumor nodules with which precise quantitative analysis could be performed by no means. In the present investigation, analysis in various aspects of X-radiation effects on ascites tumors demonstrated clearly that the slower growing tumor is more radiosensitive than the faster growing tumor, and that, the greater polymorphism a tumor shows, the more damages it receives from X-radiation; not all of the tumors of higher growth velocity show more polymorphism. These results do not support Bergonié-Tribondeou's law.

Summary

Twenty different strains of the rat ascites hepatoma were used in the present study, in an attempt to find whether, by means of comparison of the X-ray dose necessary to kill 10^7 tumor cells of each tumor strain, the individual difference in radio-

sensitivity existed among these tumors which were naturally all common in the normal ancestral cell, the liver cell; and if it existed, to know what biological characteristics of tumors it was related to. The results obtained are as follows:

1) Even the tumors derived from one and the same ancestry never did agree with each other in their radiosensitivity.

2) The radiosensitivity of tumors is related to their growth velocity. The slower growing tumors are more radiosensitive than the faster growing tumors. This result does not support Bergonié-Tribondeau's law.

3) X-radiation induced more remarkable damages in the tumors showing more marked polymorphism of cells than in the tumors consisting of uniform cells.

4) No correlation was found between the radiosensitivity of tumors and the sensitivity of them to HN_2 derivatives.

〔The major points of the present study have been reported in the 17th General Meeting of the Japanese Cancer Association, Chiba, Japan, November 9, 1958 (12)〕.

Acknowledgements

The author wishes to express his cordial thanks to Prof. Tomizo Yoshida, Director of this Institute and Department of Pathology, University of Tokyo, for his invaluable instruction and criticism during the course of this investigation. The author is grateful to Drs. H. Isaka, K. Nakamura, S. Odashima, T. Kurata, and H. Satoh, Research Members of this Institute, for their personal guidance, and to Prof. H. Sato, Department of Pathology, Fukushima Medical College, for his criticism and advice. The author wishes to acknowledge the assistance of Miss T. Watanabe and all other technicians of this Institute. He is also grateful to Prof. T. Miyagawa and his staff of Department of Radiology, University of Tokyo, for their kind suggestions and cooperations.

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[GANN, 51, 47~53; March, 1960]

THE CYTOLOGICAL EFFECT OF CHEMICALS ON TUMORS.[1] VIII. OBSERVATIONS ON CHROMOSOMES IN A GASTRIC CARCINOMA TREATED WITH CARZINOPHILIN[2]

(Plate I)

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Cytological investigation inquiring into the action of anti-tumor agents upon tumor cells is one of the important challenges in an analysis of the fundamental problems of cancer chemotherapy. Many ascites tumors of rats and mice have been used for this purpose with interesting results for understanding of the mechanism of tumor-cell damage.

Recently several anti-tumor agents have been in practical use for tumor patients. Many clinical and chemotherapeutic reports have been published by medical investigators, but there are few from the cytological viewpoint on the effect of the anti-tumor agents in human tumors, due probably to the technical difficulties in handling of human tumor cells.

Current studies of chromosomes in several human tumors have made possible the morphological analysis of chromosomes to a considerable extent (Ising and Levan 1957, Makino, Ishihara and Tonomura 1959). Carzinophilin, one of the antibiotics (Hata, Koga, *et al.* 1954) was found to be rather effective for certain human tumors. The present author has an opportunity to study the cytological effect of Carzinophilin on a gastric carcinoma with special reference to the effect on the chromosomes of the tumor cells.

The author wishes to express his cordical thanks to Professor Sajiro Makino for his kind direction and for improvement of the manuscript for publication. The author is also indebted to Dr. Y. Okuda, Department of Surgery, Hokkaido University Hospital, for the collection of the material for study.

Material and Methods: The patient bearing a gastric carcinoma was a woman, 46 years old. The tumor was first found in her stomach by the clinical examination

(1) Beginning with this article and hereafter, "tumors" will be used in the main title of this series of studies in order to include various kinds of neoplasm, because the term "ascites sarcomas" previously used indicates tumors of limited sorts.

(2) Contribution No. 458 from the Zoological Institute, Faculty of Science, Hokkaido University, Sapporo, Japan.

Supported by a grant-in-aid to Dr. S. Makino from the Scientific Research Fund of the Ministry of Education.

at the Hokkaido University Hospital in January 1959. The tumor growth was rather active, showing metastasis to many parts of the stomach, and producing a rich serous fluid in the patient's peritoneal cavity. Histological examination of the tumor proved in to be a typical carcinoma simplex. During the period from the 16th of February to the 9th of March, the patient was treated with 2,000 to 5,000 units of Carzinophilin, every day or every other day. Finally she received 99,000 units of Carzinophilin in total amount. Before the first sampling for this study which was made on 2nd March, this patient had received 55,000 units of Carzinophilin. The 2nd sampling was made on the 14th March, being 5 days after the final treatment with Carzinophilin. The 3rd sampling was made on the 19th March and the 4th on the 1st April. The patient died on the 10th April. The details of sampling data are given in Table 1.

Table 1. Data on Carzinophilin-treatment and samplings.

Data	Carzinophilin treatment	Total amount of Carzinophilin	Sampling
Feb. 11	(operation)		
Feb. 16	2,000	2,000	
⋮	⋮	⋮	
March 2	5,000	55,000	1st sampling
⋮	⋮	⋮	
March 9	5,000	99,000	
March 14			2nd sampling
March 19			3rd sampling
April 1			4th sampling
April 10	(died)		

The peritoneal fluid with suspended tumor cells drawn directly from the patient into small bottles was centrifuged for 4 minutes at 1,200 rpm. in order to collect tumor cells. The preparation for the chromosome study was made according to a water pre-treatment squash method with acetic dahlia staining. For details one may refer to the paper published by Makino, Ishihara and Tonomura (1959).

Observations

1. Frequency of the tumor cells below and above tetraploidy.

The observations revealed that the chromosome number varied within a considerably wide range from diploidy to over octoploidy in the material from earlier samplings (Fig. 6). Frequencies of the cells with less and more than 92 chromosomes were observed and summarized in Table 2. The cells with chromosome numbers over tetraploidy occurred at 57.1 per cent in the first sampling (Fig. 7), with those below tetraploidy occurring at 42.9 per cent. The 2nd and 3rd samplings gradually decreased in cell-populations. In the 4th sampling the cells over tetraploidy disappeared in the peritoneal fluid, while in striking contrast, those below tetraploidy showed a gradual increase. The data are given in summary in Table 2. The distribution of

chromosome numbers in the cells below tetraploidy is to be discussed in some detail below.

Table 2. Frequency distributions of the tumor cells below and above tetraploidy in four samplings.

	$>\pm 4n$	$<\pm 4n$	Total
1st sampling	39 (42.9%)	52 (57.1%)	91
2nd 〃	16 (59.3%)	11 (40.7%)	27
3rd 〃	29 (76.3%)	9 (23.7%)	38
4th 〃	34 (100%)	0	34
Total	118	72	190

2. Abnormalities in the chromosomes.

Morphological response of the tumor cells to Carzinophilin resulted in the production of many kinds of chromosomal abnormalities. These abnormalities were observed rather frequently in highly ploid cells. Frequently the chromosomes showed irregular elongation into atypical chromonemata (Fig. 8). In some chromosomes, the elongation appeared in a part near their centromere. Chromosomes with a bead-like appearance were also found in many highly ploid cells (Fig. 9). Stickiness and coalescence of chromosomes and chromatid breaks were rather common in the tumor cells of various types. Further, chromosomes showing somatic translocation of various types were observed in the first sample (Figs. 10–11).

It is interesting to note that similar types of chromosomal abnormalities were found by Hori and Sasaki (1958) to occur in normal and neoplastic cells *in vitro* following treatment with Carzinophilin, and by Awa (1958) in the MTK–sarcoma III of rats after Carzinophilin treatment.

3. Chromosome-number distribution in the cells below tetraploidy.

Exact counting of chromosome numbers was made in the cells with chromosomes less than 92 in number which were derived from four samplings: 39 cells were counted in the 1st sampling, 16 cells in the 2nd, 29 cells in the 3rd, and 34 cells in the 4th. Cells under study were 118 in total.

As given in Figure 1, the chromosome number scattered within a wide range from 39 to 90, with the most frequent value lying in near-triploid regions (Figs. 12–15). In every sample, the chromosome-number distribution was characterized without exception by a marked peak at 62. As already mentioned, the 1st sample was taken on the 2nd of March from the patient while Carzinophilin was being applied, whereas the 2nd to 4th samplings were made after the stoppage of the treatment. All the samples here examined showed tumor cells remarkable for their showing of a distinct stem-line number of 62. The situation here presented is sufficient to indicate that the stemline chromosome-number has persisted without change during the period when

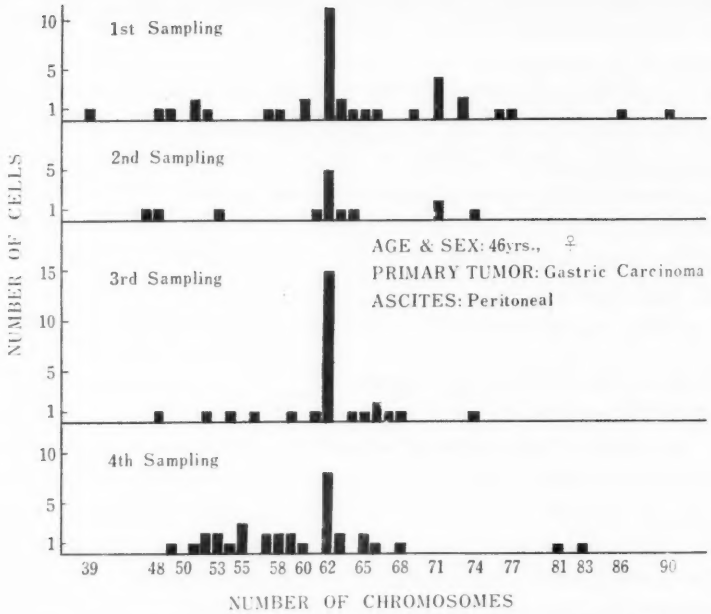


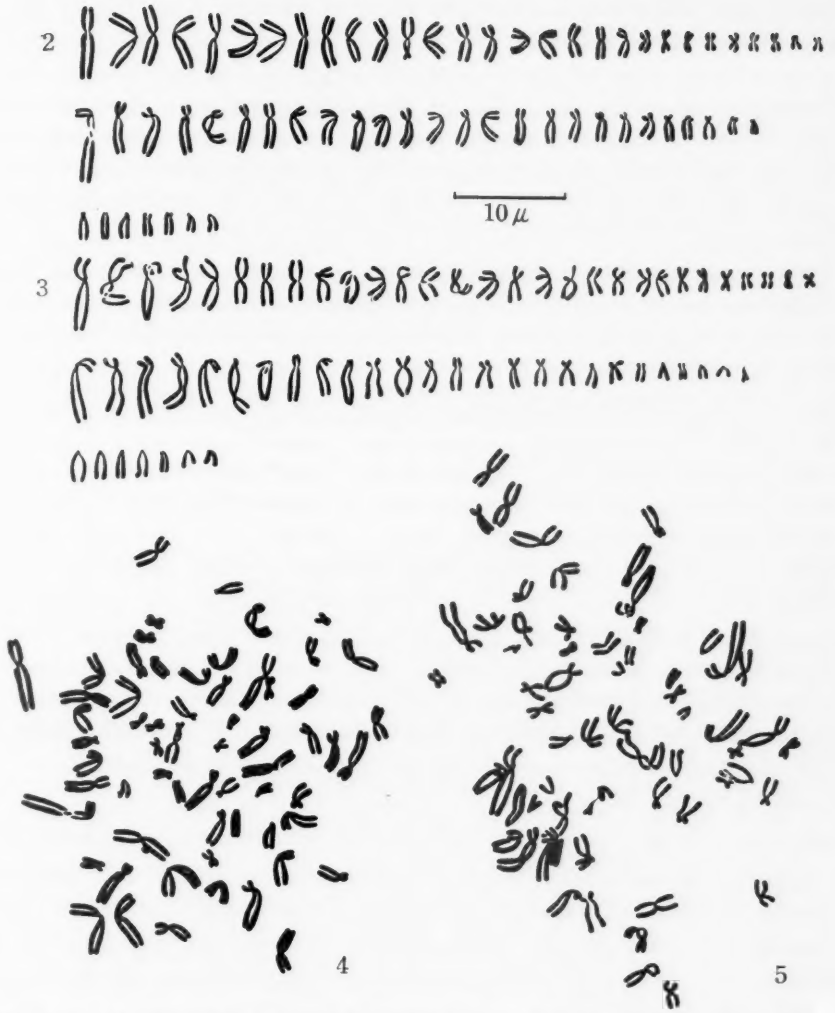
Fig. 1. Diagram showing the chromosome-number distribution of tumor cells below tetraploidy in four samplings.

the four samplings were made.

4. Morphology of the chromosomes.

As stated above, the distribution of the chromosome-number of this tumor was characterized by a hypotriploid cell with 62 chromosomes in each sampling. Idiogram analysis was made in seven cells having distinctly 62 chromosomes. The idiograms analysed are presented in Figures 2 and 3, and in Figures 4 to 5. According to Tjio and Levan (1956), the chromosome complements were classified into three groups as follows; M type with median or submedian centromere, S type with a subterminal centromere, and T type with a terminal centromere. In each type, the individual chromosomes were arranged in order of their decreasing sizes. Idiogram analysis indicated that every cell with 62 chromosomes contained 29 M-type chromosomes decreasing from 6μ to 1μ in length, 26 S-type chromosomes ranging from 5μ to 1μ, except the longest one which was about 7μ in average length, and the remaining 7 T-type chromosomes varied in length from 3μ to 1μ. The longest S type chromosome shows a ratio of long arm to short arm at 3 to 1. It is evident that the stemline idiogram of this tumor was represented by a formula, 29 (M)+26 (S)+7 (T).

The stemline idiogram of this tumor was marked specially by the presence of an outstandingly long S-type chromosome. The existence of a similar S-type chromosome



Figs. 2–3. Idiogram analysis in two hypotriploid stemline cells, 62 chromosomes in each.
Figs. 4–5. Camera lucida drawings of the metaphase chromosomes, corresponding to 2–3.

has been noted in a mammary carcinoma (Wakabayashi and Ishihara 1958) and in a gastric carcinoma (Makino, Ishihara and Tonomura 1959).

Discussion

The present investigation after the application of Carzinophilin showed the chromo-

some-number distribution to cover wide range of variation from diploidy to high polyploidy in earlier samplings. Unfortunately the chromosomes were not observed in the samples before the Carzinophilin-treatment, but it is most probable that the drug-application caused the production of mitotic irregularities of various types, together with the appearance of highly ploid cells. Carzinophilin-treatment in normal and neoplastic cells in tissue culture by Hori and Sasaki (1959), and in the MTK-sarcoma III of rats by Awa (1958) was observed to produce mitotic abnormalities of a similar type.

Working on the effect of podophillin on rat ascites tumors, Makino and Tanaka (1953a) reported that some of the tumor cells remained unaffected and formed a source of a renewed malignant growth after their proliferation. A similar situation was found to occur in several other rat ascites tumors following the application of anti-tumor agents (Makino and Tanaka 1953b, Tanaka, Kano, *et al.* 1955, Sasaki 1956, Awa 1959).

In the present study, exact counting of the chromosome numbers particularly in tumor cells below tetraploidy showed a variation ranging from 39 to 90, with a modal ploidy in the near-triploid region. In every sample herein examined, the modal chromosome number was characterized by a distinct stemline number of 62. This indicates that the stemline cells of this tumor have persisted without change during four successive samplings here undertaken. Further, the stem-cells characterized by 62 chromosomes gradually increased in number in the 3rd and 4th samples which were obtained after the stoppage of Carzinophilin-treatment. The evidence here presented implies that the stemline cells of this tumor have remained unaffected by the drug and formed a cause of tumor growth through their proliferation.

Summary

Chromosomal conditions in a human gastric carcinoma treated with Carzinophilin were observed in the present study. Distribution of chromosome numbers in four successive samplings showed a wide range of variation from hypodiploidy to above octoploidy. Various chromosome abnormalities were produced, especially in highly ploid cells.

In every sample observed here, the chromosome-number distribution was characterized by a marked mode at 62. It seems probable from the above evidence that the stemline cells with 62 chromosomes have remained unaffected by the drug, maintaining their individuality through the Carzinophilin-treatment.

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Explanation of Plate I

All figures are photomicrographs of the chromosomes in a human gastric carcinoma. (Figures 1~5 in the text).

Fig. 6. Tumor cells with various ploidy in the peritoneal fluid. ×140.

Fig. 7. Tumor cells with over 100 chromosomes. ×350.

Fig. 8. Chromosomes showing irregular elongation of the chromonemata. ×1200.

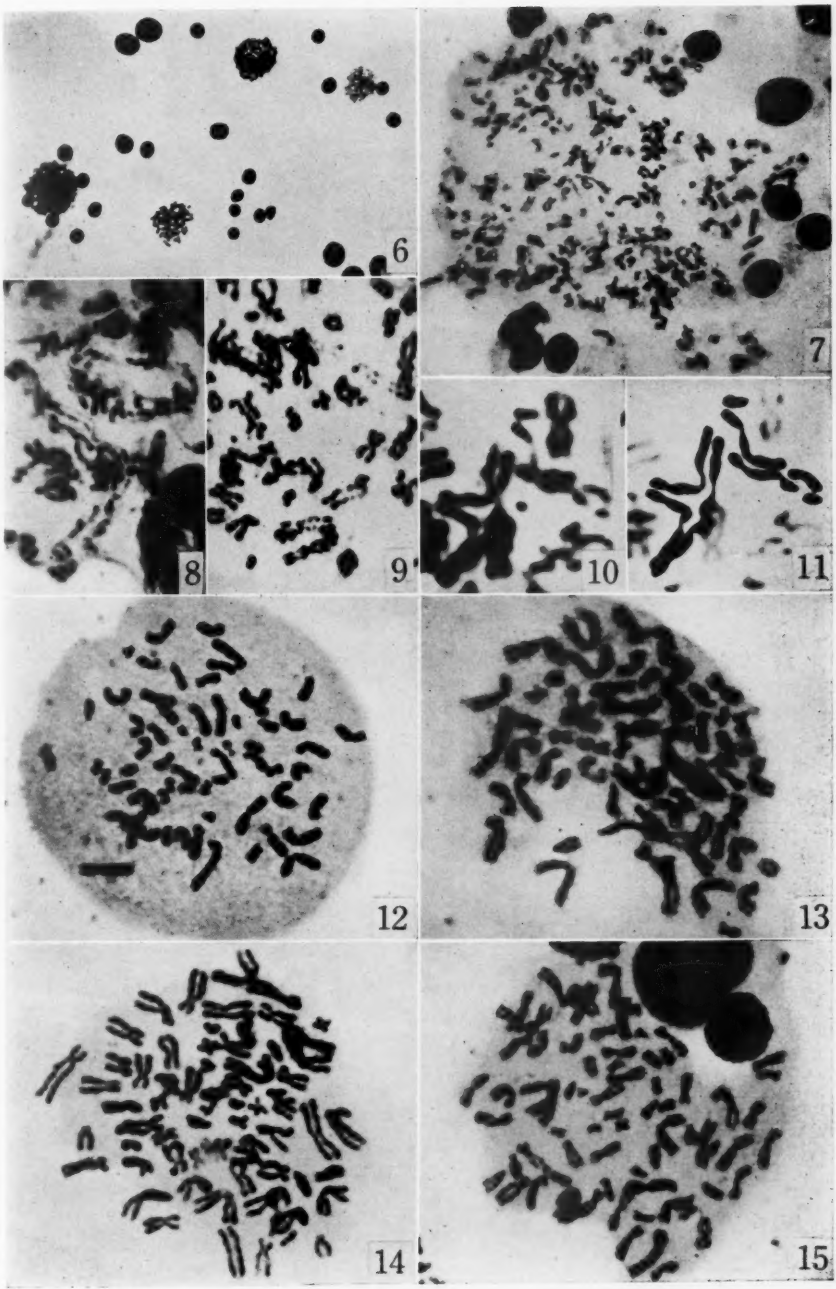
Fig. 9. Chromosomes with a bead-like appearance. ×1200.

Fig. 10. Somatic translocation of chromosomes. ×2500.

Fig. 11. Traces of Fig. 10.

Figs. 12–15. Metaphase chromosomes. ×1000.

12–13. 62 chromosomes in each. 14. 68 chromosomes. 15. 71 chromosomes.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[GANN, 51, 55~65; March, 1960]

CHROMOSOMAL ALTERATION AND THE DEVELOPMENT OF TUMORS. III. CHANGE IN PLOIDY OF THE STEMLINE CHROMOSOMES IN A MOUSE SARCOMA IN CONNECTION WITH A TRANSPLANTABILITY SHIFT[1)2)]

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The stemline concept established in transplantable ascites tumors of rats and mice indicates that the most frequently occurring tumor cells, representing a characteristic mode of chromosome numbers and a particular karyo-idiogram, form a stem lineage of cells which mainly contribute to neoplastic growth (Makino 1952, 1957). Available data collected during the last several years show that the constancy of the stemline, though it is rather pronounced, is not necessarily permanent, and that the ascites tumors during serial transfers have undergone numerical and structural chromosome changes with changes in the type of disease (Makino and Sasaki 1958). Changes of the mode from diploid to tetraploid in mouse ascites tumors have been reported by Levan and Hauschka (1953), Hauschka (1953, 1956), Klein (1955) and Sachs and Gallily (1955).

The MY-mouse sarcoma herein dealt with is one of the transplantable spindle-cell sarcomas, spontaneous in origin. In the course of serial transfers, a change of the stemline chromosome-number took place from diploidy to tetraploidy. In the present paper, the authors wish to report in some detail the course of the chromosomal change during serial transfers resulting in the shift in transplantability.

Before going further, the authers wish to acknowledge here their indebtedness to Professor Sajiro Makino, Hokkaido University, for his keen interest in this subject and his kind revision of the manuscript. Further they are also indebted to Mr. Yoshinori Kurita for friendly assistance.

Material and Methods

MY-mouse sarcoma here under consideration is one of the transplantable sarcomas;

1) Contribution from the National Institute of Genetics, Misima, Japan, No. 297.
2) Aided by a grant from the Science Research Fund of the Ministry of Education.
3) Present address: Radiation Research Laboratory, Medical Dept., Hokkaido University. Sapporo, Japan.
4) Present address: Pathology Laboratory, Medical Dept., Kyushu University, Fukuoka, Japan.

it spontaneously developed in a so-strain mouse (Yosida 1952). It has been up to date transferred serially as a solid tumor to several strains of mice for 120 transplant generations.

The mice of so-strain are of hybrid origin between a SMA-mouse and a non-inbred albino mouse common in Japan. SMA- and DM-mice* were established by sister and brother matings in the animal breeding house of Hokkaido University in 1944 by Yosida and Makino (1954). Among mice used for tumor transfers, b-mice were originated from hybrids between common albinos and wild mice (Yosida and Makino 1954), and D103-mice were derived from DM-mice, while S4-, SL-, SF- and SK-mice have their origin in SMA-mice (Ishihara and Yosida 1958). The dd-mice started from an albino mouse common in Japan and were inbred in this institute for about 10 generations. The mice of other strains used here (Tables 2, 5 and 6) were imported from the United States.

Generally a solid tumor is a difficult material for a cytological study. The tumor tissue was removed from tumor-bearing mice and cut into small pieces, which were inserted into the peritoneal cavity of the mouse with the aid of a transplantation-needle. Bits of the tumor pulp thus inoculated were removed from the host on the third or fourth day after inoculation. For the study of chromosomes of the diploid tumor-strain, the pulp was fixed in Carnoy's solution (1 : 3) for about half an hour, and transferred to 10 and 50 per cent acetic acid leaving it for 5 minutes in each. The material has placed on a clean slide, stained by applying a few drops of acetic orcein, and squashed under a cover-slip. For the observation of the tetraploid subline, the material was pretreated with a hypotonic solution consisting of an equal volume of N/1,000 $NaCl_2$ and N/100 $CaCl_2$ (Yosida and Ogawa 1956); the cells were subjected to the acetic orcein squash technique.

Results of experiments and observations

1) Transplantability in early transplant generations: The transplantability of the MY-mouse sarcoma in early transplant generations was studied by Yosida (1952), Ishihara and Yosida (1954) and Yosida and Ishihara (1955). According to Yosida (1952) this tumor was transplanted successfully into SMA-, b- and so-strain mice in the course of 8 transplant generations, while the DM-strain showed negative transplantability in only one mouse used. The transplantability to DM-, SMA- and so-mice increased between the 13th to 24th transplant generation, while that to b-mice was negative (Table 1 and Ishihara and Yosida 1954).

Further the transplantation of the tumor was made employing mice of various strains such as DM, SMA, SK and C3H, A, DBA/Ma, SWR, Swiss albino, and CBA,

*so-, SMA-, DM- and b-strains have been described as SO-, S-, D- and B-strains, respectively, in previous reports (Yosida 1952 and some others)

Table 1. Transplantability of the MY-mouse sarcoma according to mouse strains and number of transplant generations

Strain \ Transplant generation	1–8	13–24
SMA	32.4% (71)	70.6% (17)
DM	0 (1)	59.3 (32)
b	74.4 (47)	0 (13)
so	44.1 (34)	52.6 (36)

The figures in parenthesis denote the number of mice used.

all of them imported from the United States. Tumors of the 32nd to 38th transplant generations were used for experiment. The results are summarized in Table 2. The transplantability to C3H-, A-, DBA/Ma-, SWR-, Swiss albino- and CBA-mich was entirely negative, whereas SMA-, SK- and DM-mice showed transplantability of 62.0, 87.5 and 76.5 per cent, respectively. The growth feature of tumors after transplantation differs according to the mouse strain used. For instance, tumors grafted into SK-strain mice showed an active growth, and the host died of the tumor 19 to 25 days after inoculation. The DM-strain mice died of tumor 24 to 48 days after inoculation. Although transplantation to C3H-, A-, DBA/Ma-, SWR-, Swiss albino- and CBA-mice was negative, grafts inoculated into C3H- and DBA/Ma mice showed atrophy after a temporary developmental change. The transplantation to C3H- and CBA-mice was wholly negative even after 80 trials. As shown in Table 2, the transplantability into D103- and S4-mice was very high.

Table 2. Transplantability of the MY-mouse sarcoma to mice of various strains in the course of 32nd to 38th transplant generations.

Strain	No. of mice used	Results of transplantation		% of positive results
		Positive	Negative	
SMA	53	33	20	62.2
SK	16	14	2	87.5
S4	62	62	0	100
DM	34	26	8	76.5
D103	51	47	4	92.2
C3H	70	0	70	0
DBA/Ma	6	0	6	0
SWR	11	0	11	0
Swiss albino	8	0	8	0
A	10	0	10	0
CBA	3	0	3	0

As stated before, C3H-mice showed a negative result for transplantation of this tumor. In order to know the number of histocompatibility genes of this tumor, the

transplantability was studied in F_1 hybrids between D103- and C3H-mice. As shown in Table 3, transplantation to all of the F_1-mice was positive. In the light of this fact, it seems that the transplantability of this tumor is controlled by dominant genes (H-genes). Then, the transplantability was examined in F_2 and in the back-cross ($C3H \times F_1$). The results showed that 57.3 per cent of F_2 and 30.7 per cent of RF_1 hybrids showed positive transplantability. Based on the above results, it is most probable that two H-genes are concerned with the transplantability of this tumor.

2) Chromosome pattern of tumor cells in early transplant generations: Although solid tumors are generally a difficult material for chromosome study, the authors have some data on the chromosomes of this tumor in solid form in early transplant generations. chromosome numbers observed in 20 cells showed a variation from 36 to 75,

Table 3. Transplantability of the MY-mouse sarcoma in F_1 (D103×C3H), F_2 and backross mice (F_1 to C3H).

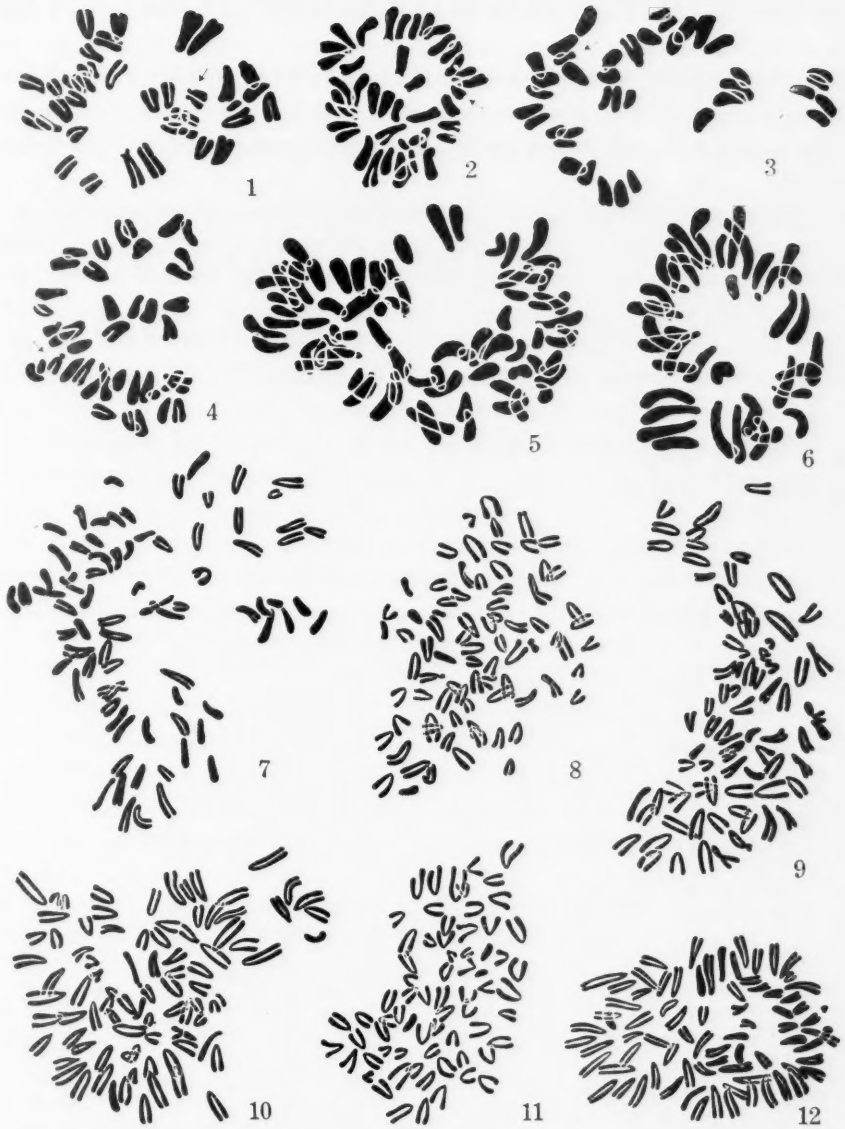
Cross	No. of mice used	Results of transpl.		% of positive results
		Positive	Negative	
$(D103 \times C3H)F_1$	91	91	0	100
$(D103 \times C3H)F_2$	68	31	29	57.3
RF_1 $(C3H \times F_1)$	65	20	35	30.7

cells having 39 chromosomes being most frequent: 19 among 20 cells had 36 to 44 chromosomes, only one cell showing the hypotetraploid chromosome-number of 75 (Table 4). There were observed cells showing abnormalities such as multinuclei, atypical arrangement of chromosomes and abnormal swelling of chromosomes. Interspersed with cells showing mitotic abnormalities, there was present a population of tumor cells which contained a well-balanced subdiploid complex of chromosomes and occurred at a high frequency. There is no doubt that those subdiploid cells are stem cells which contribute primarily to the growth of this tumor. Frequent examinations of the chromosome number in the course of transplant generations showed that no visible change occurred at least until the 88th transplant generation.

Table 4. Chromosome-number distribution in the MY-mouse sarcoma in early transplant generations.

No. of chroms.	36	38	39	40	41	42	44	75	Total
No. of cells obs.	1	2	7	4	2	2	1	1	20

Though the vast majority of chromosomes of this tumor are of rod type (Figs. 1-6), there was observed in the complex one small-sized chromosome characterized by a subterminal constriction. Such chromosomes are indicated by arrows in Figures 1 to 4. Serial alignments of chromosomes in three cells are shown in Figures 13 to 15 in which 42, 41 and 39 chromosomes are shown, respectively. There is no evidence



All figures are camera-lucida drawings at magnification of 2000×. Figs. 1 to 6 and Figs. 7 to 12 are reduced to 1/2 and 1/3, respectively.

Figs. 1–6. Tumor cells of the MY–mouse sarcoma in early transplant generations. Figs. 1 and 3, 39 chromosomes. Fig. 2, 42 chromosomes. Fig, 4, 41 chromosomes. Fig. 5, 75 chromosomes. Fig. 6, 40 chromosomes.

Figs. 7–12. Tumor cells of the MY–mouse sarcoma between the 94th and 100th transplant generations. Figs. 7 and 8, 79 chromosomes. Fig. 9, 81 chromosomes. Fig. 10, 88 chromosomes. Fig. 11, 69 chromosomes. Fig. 12, 75 chromosomes.

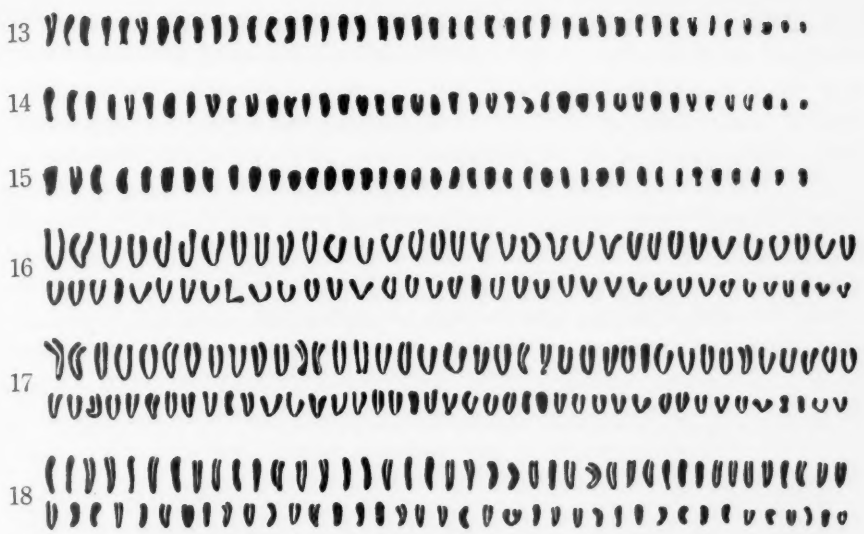
for the presence of a large V-shaped element which is commonly observed in tumor cells of the Ehrlich ascites tumor and the Takizawa quinone carcinoma of mice (Yosida 1954). Comparison between normal somatic cell and tumor cells reveals that most of the tumor chromosomes resemble in general outline those of the normal cells, while the mode for the former is 39 containing a small element of subterminal structure.

3) Shift of transplantability in recent transplants: It was formerly described that the present tumor failed to grow in mice of various strains received from the United States, while its transplantation was successful to mice of some Japanese strains. The transplantability of this tumor was examined again in mice of various strains such as DM, D103, SMA, S4, DBA/Ma, C3H, A, Swiss albino, C57BL, C57L, C58, dba, sps, and dd from the 94th to 100th transplant generation. The results indicated that all mice here examined showed positive transplantability of 80 to 100 per cent (Table 5), except DM-mice in which it amounted to 18.8 per cent. In early transplant generations the transplantability to DM-mice was 76.5 per cent, but in the 94th to 100th transplant generations it was reduced to 18.8 per cent. The average life span of 16 DM-mice was found to be 43 days in the above transplant generations. This is the longest life span in all mice here examined. D103-mice which originated from

Table 5. Transplantability of the MY-mouse sarcoma according to various strains in 94th to 100th transplant generations.

Strain	No. of mice used	Results of transpl.		% of positive transpl.	Life span (day)	
		Positive	Negative		Aver.	Range
DM	16	3	13	18.8	43	42-43
D103	30	30	0	100	33	21-47
SMA	12	10	2	83.3	28	16-33
S4	19	19	0	100	31	23-40
DBA/Ma	14	11	3	78.6	33	16-64
C3H	12	10	2	83.3	38	31-49
A	14	12	2	85.7	36	23-42
Swiss albino	12	12	0	100	28	20-39
C57L	11	11	0	100	21	12-32
C57BL	11	11	0	100	31	20-44
C58	17	16	1	94.1	31	19-48
dba	11	11	0	100	28	20-38
sps	9	8	1	88.9	32	24-41
dd	19	18	1	94.7	33	24-43

DM-mice showed a transplantability of 100 per cent in the same transplant generation. The average life span of D103-mice was 33 days. SMA-mice showed 83.3 per cent transplantability of this tumor, while S4-mice originated from SMA-mice showed 100 per cent transplantability. The transplantability into DBA/Ma-, C3H-, A-, Swiss



Figs. 13–18. Serial alingment of chromosomes in MY-mouse sarcoma cells. Figs. 13 to 15, near diploid cells. Figs. 16 to 18, near tetraploid cells. Figs. 13, 14, 15. 16, 17 and 18 from Figs. 2, 4. 3, 11, 8 and 7, respectively.

albino-mice increased from zero to 78.6, 83.3, 85.7, 100 per cent, respectively. The transplantability to mice of other strains such as C57L, C57BL, C58, dba, sps, and dd varied from 88.9 to 100 per cent. Tumors from the 115th to 118th transplant generations were transplanted to mice of various strains. As shown in Table 6, D103-, SF-, SL- and C57BL-mice showed 100 per cent transplantability, while CBA-, Swiss albino- and C3H-mice had a transplantability of 90.5, 94.1 and 75.0 per cent, respectively. Mice of NH/He strain showed a lower transplantability of 26.7 per cent. It seems probable that the NH/He mice have a considerable resistance to the present tumor.

Table 6. Transplantability of the MY-mouse sarcoma to mice of various strains in 115th to 118th transplant generation.

Strain	No. of mice used	Results of transpl.		% of positive transpl.	Average life span (Day)
		Positive	Negative		
D103	17	17	0	100	44
SF*	15	15	0	100	37
SL*	16	16	0	100	40
C3H	20	15	5	75.0	34
Swiss albino	16	15	1	94.1	42
CBA	21	19	2	90.5	29
NH/He	15	4	11	26.7	75

* SF and SL strains are derived from SMA-strain.

4) Change of ploidy in tumor stem-cells: It was observed that following the 94th transplant generation the present tumor showed a wide-spread transplantability to mice of various strains herein examined. Specially noticeable is the fact that the stemline chromosome-number showed a change from subdiploidy to near-tetraploidy in association with the shift in transplantability. As stated before, this tumor was characterized by the near-diploid chromosome-number in early transplant generations, while in the 94th to 100th transplant generations, 92.5 per cent of tumor cells showed near-tetraploidy (Table 7). The chromosome number observed in 23 tumor cells

Table 7. Frequency of diploid (±2n), tetraploid (±4n) and octoploid cells (±8n) in the MY-mouse sarcoma in 94th to 100th transplant generations.

Ploidy	Diploid (±2n)	Tetraploid (±4n)	Octoploid (±8n)	Total
No. of cells obs.	2 (3.0%)	62 (92.5%)	3 (4.5%)	67

Table 8. Chromosome-number distribution in the MY-mouse sarcoma in 94th to 100th transplrat generations.

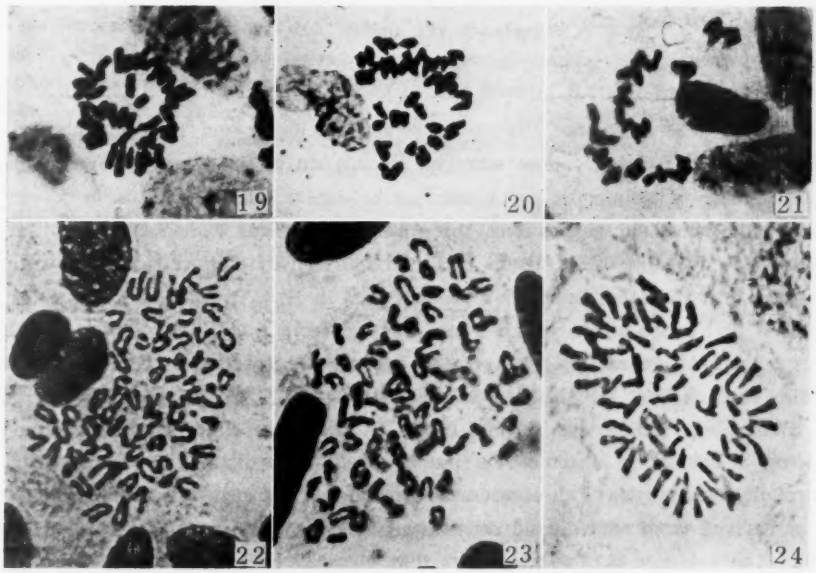
No. of chroms.	69	76	77	78	79	80	81	88	Total
No. of cells obs.	2	2	4	4	6	2	2	1	23

ranged from 69 to 88 with the mode at 79 (Table 8). Idiogram analysis showed that the majority of chromosomes are of rod-shape. No large elements of V- or J-shape or small dot-like chromosomes were found which are common in the Ehrlich ascites tumor and the Takizawa quinone carcinoma (Figs. 7-12 and 22-24). Serial alignments of chromosomes in three tumor cells are shown in Figures 16-18: almost all chromosomes are characterized by terminal centromeres, the remaining one or two small bodies having a subterminal constriction. In the diploid tumor cells observed in early transplant generations, a small subterminal chromosome was frequently observed. In the tetraploid tumor-line, the mode of chromosome numbers was 79, and tumor cells having 77 and 78 chromosomes ranked secend in frequency. Based on the above results, it may be assumed that the tetraploid tumor cells derived from the diploid ones through a mechanism of reduplication of chromosome number.

Discussion

Change from diploid to tetraploid chromosome number has been reported for several transplantable mouse tumors. According to Levan and Hauschka (1953), most of the diploid tumor contain exceptional polyploid cells as a result of endomitotic reduplication; in general, near-diploid cells are found at about 5 per cent in a neoplastic population. Such a population tends to remain true to the original chromosome number, but these near-tetraplid cells occasionally become preponderant and dominate over the diploid ones. This type of shift was reported by Hauschka (1953), Klein (1955), Sachs and Gallely (1955) and Hauschka *et al.* (1956) in TA3 ascites carcinoma,

S3A carcinoma, C3H sarcoma and 6C3HED lymphoma. Hauschka (1953) found that a spontaneous change in chromosome-number mode occurred in the TA3 ascites carcinoma resulting in the loss of the tumor's rigid host-specificity. According to Klein (1955), the S3A carcinoma, a solid mammary canncer, changed its chromosome number from 40 to 72 in the course of transfomation into an ascites form. Hauschka *et al.* (1956) found that the 6C3HED ascites lymphoma was transplanted successfully into C3H mice. After serial transplantations to refractory mice of Swiss albino strain, this tumor was transferred to DBA/2 mice. The DBA/2 adapted subline had near-tetraploid chromosomes irrespective of the home strain of this tumor having a diploid mode. According to Hauschka (1956), such an expanding of transplantability or loss of strain specificity of tetraploid cells may be explained by the phenomenon of immunoselection. Sachs and Gallily's observations on C3H sarcoma resulted in that the chromosome duplication and loss of strain specificity occurred in the solid tumor during a relatively short period. In heterologous transplantations a change of



Figs. 19–24. Photomicrographs of tumor cells in the MY-mouse sarcoma. All figures are taken with the aid of "Leitz-Makam" at magnification of 1000× and enlarged to 2 times. Figs. 19 to 24, the same as Fig. 2, 4, 3, 11, 8 and 12, respectively.

chromosome number was found by Ising (1958) in a mouse ascites tumor. It was shown by Makino and Sasaki (1958) in the Yoshida sarcoma, an ascites tumor of rats, that the numerical and structural alterations were important in starting new types of tumors, and that the development of tumors with a shift in the type of tumor properties took place in association with chromosomal changes in the stem-cells

resulting in the formation of sublines. In the case of the MY-mouse sarcoma, a change in chromosome-number mode occurred also in the course of transplantations of a solid tumor. As stated on the foregoing pages, MY-mouse sarcoma had near-diploid chromosome-number mode until the 88th transplant generation, while at the 94th generation, the number-mode changed to near-tetraploidy. It is apparent that very pronounced change in chromosome-number mode occurred in tumor cells during 6 transplant generations. Further, it is evident that the change in transplantability of this tumor has occurred in association with the alteration of the chromosome-number mode.

Such inverse relationship between ploidy and host-specificity has been found by Hauschka and Levan (1953) in 16 transplantable mouse tumors. Generally speaking, tumors having a diploid chromosome-set have a narrow range, while those having a tetraploid set have a wider range of host specificity. The results of the present study seem to offer an appreciable example illustrating the inverse relationship between ploidy and host-specificity.

Summary

In the present study the change in strain specificity and transplantability was investigated in the MY-mouse sarcoma in relation to the alteration from diploidy to tetraploidy of stem-cells.

In early transplant generations, the present tumor was transplantable to mice of SMA, DM and some other strains and characterized by stem-cells of a near-diploid complex. On the other hand, this tumor failed to grow in mice of C3H, DBA/Ma, A, SWR and Swiss albino which were imported from the United States. After the 94th transplant generation, this tumor became transplantable to mice of various strains such as DM, D103, SMA, S4, SK, SL, SF, C57L, C57BL, C58, DBA/Ma, C3H, A, SWR, Swiss albino, sps, dd and dba. At the same time the tumor stem-cells showed a change in chromosome number from near-diploidy to near-tetraploidy. Morphological analysis of chromosomes revealed that the near-tetraploid cells seemed to be derived from near-diploid cells through the mechanism of chromosomes duplication. The evidence obtained indicates that changes have occurred in the stemline chromosomes in association with changes in tumor properties during serial transfers.

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[GANN, 51, 67~81 ; March, 1960]

ELECTRONIC STRUCTURE AND CARCINOGENIC ACTIVITY OF CONJUGATED COMPOUNDS, SUBSTITUTED AROMATIC HYDROCARBONS, HETEROAROMATIC COMPOUNDS AND AZO COMPOUNDS

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INTRODUCTION

A considerable number of approaches have been explored in an attempt to elucidate the reaction mechanism involved in the early stage of carcinogenesis. One of these is the effort to correlate the carcinogenic activity to quantum-chemical indexes, and the accumulated knowledge has led us to a better understanding of carcinogenic reaction of chemical carcinogens.

The present authors have previously found a distinct parallelism between carcinogenic activity and electronic structure of nonsubstituted polycondensed aromatic hydrocarbons (1) by using the frontier electron theory which has been established by us and proved to be one of the best methods in predicting the chemical reactivity of conjugated compounds (2, 3, 4, 5, 6).

In discussing the nonsubstituted compounds, however, we could pass without saying about the type of reaction because the theoretical indexes are entirely the same for all types of reactions, that is, for electrophilic, radical and nucleophilic reactions.*

But we are compelled to decide the type of reaction in discussing the activity of substituted aromatic hydrocarbons, heteroaromatic compounds and azo compounds, because the indexes for these compounds have not the same values for each type of reaction.

In 1955 we proposed that dimethylaminoazobenzene (DAB) and its derivatives will interact with nucleophilic group in tissue protein or enzyme in early stage of carcinogenesis (7). This is the first report that stressed the importance of nucleophilic reaction in carcinogenic action of conjugated compounds. In that paper we designated the dimethylamino nitrogen and azo linkage as principal carcinogenophore and subsidiary carcinogenophore, respectively. Thereafter we stressed the importance of nucleophilic

*In organic chemistry, the type of reaction has been classified as electrophilic, radical and nucleophilic according to the electronic behavior of reagent; when the reagent has a positive charge, zero and a minus charge, the reaction has been named as electrophilic, radical and nucleophilic reactions, respectively.

reaction in carcinogenesis (8), and pointed out the strong similarity between the aromatic hydrocarbons and azo compounds such as DAB with respect to their steric circumstances, and assumed that the binding with nucleophilic center would take place at position 2 of DAB or its derivatives (9). Furthermore, our calculations of substituted aromatic hydrocarbons, heteroaromatic compounds and azo compounds support the nucleophilic mechanism of carcinogenic reaction (9). In connection with these findings we have noticed the importance of the experimental results that nitrogen mustard and epoxides, which are carcinogenic as well as carcinostatic, react with nucleophilic group under the physiological conditions (10, 11). These knowledges have led us to the suggestion that the mechanism of all types of chemical carcinogens might include the binding with nucleophilic center which may be related in sense to the growth regulation (9).

Contradictory to our postulation, Pullman and other researchers have been considering the electrophilic reaction in carcinogenesis (12, 13, 14, 15, 16, 17). But recently some studies have begun to appear suggesting an alteration of the old line of thought that the carcinogenic reaction is electrophilic in nature. That is, in a recent report Heller and Pullman (18) postulated the nucleophilic type of reaction in carcinogenic action of DAB and its derivatives. And Mason (19), in comparing the orbital energy of polycondensed aromatic hydrocarbons with the calculated band energies of protein, has concluded that the transition of an electron from the occupied band of protein to hydrocarbon molecule would occur in the initial stage of carcinogenic action of these compounds. Although Mason has not used the word "nucleophilic", the first step of the electron transition mechanism proposed by him basically conforms to our nucleophilic mechanism.

In the present paper, the frontier electron theory was applied to the substituted aromatic hydrocarbons, heteroaromatic compounds and azo compounds with regard to the elucidation of the reaction mechanism of these compounds in carcinogenesis.

Theoretical Indexes

We are not here to review the large body of information which has been accumulated concerning the frontier electron theory. Therefore we describe briefly the theoretical indexes which have been used in that theory as measures of chemical reactivity of conjugated molecules. These are the "frontier electron density", "superdelocalizability (Sr)" and "approximate superdelocalizability (S'r)" (2, 3, 4, 5, 6). It has become clear that the position which is the most susceptible to attack is that of the highest value of these indexes, not only in the case of substitution but also in the case of addition and molecular complex formation. Frontier electrons are defined as the two electrons occupying the highest molecular orbital in the ground state in the case of reaction with an electrophilic reagent. In the case of reaction

with a nucleophilic reagent, the frontier orbital is the lowest vacant orbital of the ground state: and, in the case of reaction with a radical reagent, both the two orbitals mentioned above.

These indexes were derived from the "hyperconjugation energy ($\mathfrak{H}_r$)" which is the most fundamental quantity in our theory (6). The hyperconjugation energy at the rth atom in the molecule is given by

$$\mathfrak{H}_r \cong \left\{ 2 \sum_{j=1}^{m} \frac{-(C_r{}^j)^2}{h_t - \lambda_j} + n_h \sum_{j=1}^{n} \frac{(C_r{}^j)^2}{h_t - \lambda_j} \right\} \gamma_t{}^2$$

where n is the total number of π orbitals in the molecule, and the occupied levels are denoted by 1, 2, ...m; $C_r{}^j$ is the coefficient of the rth atomic π orbital in the jth molecular orbital; and λ_j is the coefficient in the equation $\varepsilon_j = \alpha + \lambda_j \beta$, ε_j being the energy of the jth molecular orbital, and α and β being the Coulomb and the resonance integral of a carbon atom and a C-C bond in benzene, respectively; h_t and γ_t are the Coulomb integral of the pseudo-orbital* and the resonance integral between the pseudo-orbital and the rth atom of the substrate molecule in which reaction takes place, respectively. n_h is 0, 1 or 2 according as the reagent is electrophilic, radical or nucleophilic, respectively.

According as the value of h_t is equal to α or to the frontier orbital energy, $\mathfrak{H}_r$ corresponds to nothing but the superdelocalizability or frontier electron density. Therefore, these two indexes correspond to the two extreme values of $\mathfrak{H}_r$. Approximate superdelocalizability (S'r) is equal to the value of Sr in which the contribution of frontier orbital alone is taken into consideration. S'r serves not only as one term approximation of Sr but also as an index of intermediate character between Sr and frontier electron density. In other word, S'r was expected to be a good index when h_t lies between α and the frontier orbital. It might be natural to assume that the value of h_t lies in the very proximity to α or coincides with it in ordinary organic reaction, but in some cases h_t would not necessarily coincide with α, but lie in the neighborhood of the frontier orbital. In this case S'r is expected to become a better index, and this means that the frontier electrons play a more significant role in the course of reaction. In addition to our theory, Brown's recent study (20) concerning the mechanism of aromatic substitution has made clear the special importance of frontier orbital, and indicated that in nitration of aromatic hydrocarbons the greater part of the easiness of the reaction was determined by the contribution of the frontier electrons. These circumstances may naturally be expected in other chemical reactions and also in biochemical reactions.

*In the course of reaction, the reagent approaches to the place at which the reaction takes place and in the neighborhood of the transition state, the reagent and the atom of attacked place can be regarded as a quasi-π orbital and this is called a pseudo-orbital.

Results and Discussions

a) Methyl substituted 1, 2-benzanthracene

A significant change in carcinogenic activity is seen with methyl substitution in each position of 1, 2-benzanthracene (21). Especially, the compounds with methyl substitution in the benz ring have been found to be all experimentally inactive, whereas they would have been expected to be active according to Pullman's theory (12). Not a few attempts (15, 17) have been made to obviate this difficulty. However, no satisfactory success has ever been attained.

The present authors have calculated the frontier electron density at principal carcinogenophore (f_p) of 1, 2-benzanthracene and its methyl derivatives and the experimental activity.*

Table 1. Relation between the frontier electron density at the principal carcinogenophore (f_p) of 1, 2-benzanthracene and its methyl derivatives and the experimental activity.*

compound	f_p for nucleophilic	f_p for radical	f_p for electrophilic	carcinogenic activity
9-methyl-1, 2-benzanthracene	0.443	0.241	0.342	++
8-methyl-1, 2-benzanthracene	0.419	0.244	0.331	+
5-methyl-1, 2-benzanthracene	0.396	0.274	0.335	++
6-methyl-1, 2-benzanthracene	0.375	0.293	0.331	+
3'-methyl-1, 2-benzanthracene	0.363	0.305	0.334	−
10-methyl-1, 2-benzanthracene	0.352	0.323	0.338	+++
7-methyl-1, 2-benzanthracene	0.348	0.325	0.336	+
1'-methyl-1, 2-benzanthracene	0.348	0.339	0.343	−
1, 2-benzanthracene	0.344	0.344	0.344	+
2'-methyl-1, 2-benzanthracene	0.311	0.385	0.348	−
4'-methyl-1, 2-benzanthracene	0.301	0.387	0.344	−

* The parameter used in the calculation is a usually recognized value: that is the Coulomb integrals for methyl group and the carbon to which the methyl group is attached were taken as $\alpha+3\beta$, $\alpha-0.1\beta$ respectively, and the resonance integral between the methyl group and carbon atom as β.

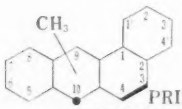


Fig. 1. Principal and subsidiary carcinogenophores of methyl substituted 1, 2-benzanthracene.

carcinogenophore (f_p) for all types of reactions, that is, electrophilic, radical and nucleophilic reactions and the results are indicated in Table 1. The site and relative positions of principal and subsidiary carcinogenophore are the same for all these compounds as indicated in Fig. 1.

From Table 1, it is obvious that a considerable degree of correlation exists between the frontier electron density for nucleophilic reaction and the carcinogenic activity. On the other hand, there is found a nearly reverse correlation with the frontier

electron density for electrophilic reaction. This is another support that the carcinogenic reaction is not electrophilic but nucleophilic in nature. For radical reaction, there is seen a complete absence of correlation between the index and the experimental activity. As was described in the previous section, frontier electron density is the best index in that case in which h_r coincides with the frontier orbital. Therefore, it is assumed that the interaction between these carcinogens and nucleophilic center in the body would proceed in such a state as the above condition is satisfied. Although the electronic character may be of primary importance for carcinogenic activity, other effects, such as permeability, diffusibility and steric effect must also operate. Especially the fact that both electron-donating and electron-attracting substituents at 10 position can transform 1, 2-benzanthracene into a more active carcinogenic compound seems to make it difficult to discuss this problem only from the standpoint of the electronic distribution. For instance, from the brief survey of the experiments on the 10 substituted 1, 2-benzanthracene it does seem likely that the moderate size of the substituent is very favorable for the carcinogenic activity. Thus the extremely great tumor-producing activity of 10-methyl 1, 2-benzanthracene may be explained if we take this effect into consideration.

An exception in Table 1 is 3′-methyl compound; since the 3′ and 4′ positions of these compounds have been considered as the places at which metabolism or detoxication occurs (22), inactivity of 3′-methyl compound might be understood in connection with this metabolic process. The values of frontier electron density for 3-methyl and 4 methyl compounds have not been included in the table, because the principal carcinogenophore of these compounds is blocked by methyl group, so that the comparison of these values with those of other compounds seems to be meaningless.

It may be worth considering here about the reason why 3-methyl and 4-methyl compounds, in which the principal carcinogenophore is blocked by methyl group, are none the less carcinogenic. In this case, substitution reaction at two adjacent carbon atoms of principal carcinogenophore is really difficult to take place simultaneously. However, the substitution at that carbon atom which is not blocked by methyl group can easily be considered to take place. In fact, by the experiment of Heidelberger, the binding *in vivo* reaction at 3, 4 carbons of 1, 2, 5, 6-dibenzanthracene or at one of these carbon atoms was proved (23, 24). Or this problem is also explicable if we adopt other types of reactions, such as addition reaction or molecular complex formation, in which the existence of sterically larger group than hydrogen atom may not be greatly preventing.

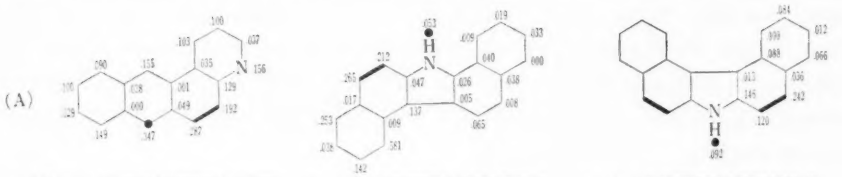
As Coulson pointed out (25), it is difficult to give a clear explanation by the existing theories as to the reason why 1′, 2′, 3′ and 4′ methyl compounds have no carcinogenic activity, because, according to these theories methyl substitution at these positions leads to an increase of the total π electron density at the K-region compared with

that of nonsubstituted 1, 2-benzanthracene which was found to be a moderate carcinogen. It is seen in Table 1 that the values of the frontier electron density of 2′, or 4′ methyl compounds are far below those of nonsubstituted compounds, and 1′-methyl compound has the same order of frontier electron density. In this sense the parallelism between the frontier electron density and the experimental activity is much more complete than that which exists between the activity and the quantities of other theories. The role of the subsidiary carcinogenophore in carcinogenesis may be of a secondary significance as in the case of nonsubstituted aromatic hydrocarbons, because there exists no correlation between the values of the frontier electron density at subsidiary carcinogenophore and the experimental activity.

b) Heteroaromatic Compounds

In general, an aza replacement which converts an aromatic hydrocarbon to a heteroaromatic molecule causes a decrease of carcinogenic activity (16). For example, 1′-aza-3, 4-benzpyrene is entirely inactive whereas 3, 4-benzpyrene is one of the most potent carcinogens (21), but there seems to have been no theory giving a clear explanation on this fact.

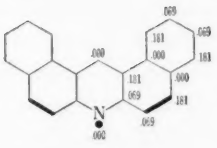
In this section we treated the nitrogen-containing compounds such as aza-derivatives of aromatic hydrocarbons, benzacridines, and benzcarbazoles. These compounds were classified to (A), (B) and (C) groups according to the same principle as in the previous paper (1). That is, the compounds belonging to the A group have both the principal and the subsidiary carcinogenophores and at the same time have sufficiently large values of frontier electron distribution at the principal carcinogenophore. They are carcinogenic. Group B compounds have the principal carcinogenophore only. These are slightly carcinogenic or noncarcinogenic, even if the values of index at principal carcinogenophore are above the critical value. Group C compounds have either the subsidiary carcinogenophore only, or are devoid of both the principal and the subsidiary carcinogenophores, or have both carcinogenophores but a small value of the index at principal carcinogenophore. They are all noncarcinogenic. The situation is seen in Figs. 2 and 3.* In these figures the values of frontier electron density for electrophilic and nucleophilic reactions are given, and the principal and



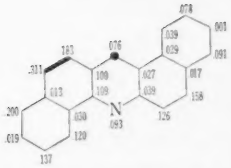
4′-AZA-1, 2-BENZANTHRACENE 1, 2, 5, 6-DIBENZCARBAZOLE 3, 4, 5, 6-DIBENZCARBAZOLE

*The value of frontier electron density for radical reaction is easily obtained from Figs. 2 and 3 because the arithmetic mean of the values for electrophilic and nucleophilic becomes the value for radical reaction.

(A)

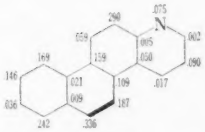


3, 4, 5, 6-DIBENZACRIDINE

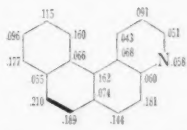


1, 2, 5, 6-DIBENZACRIDINE

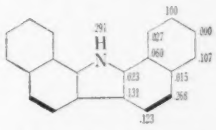
(B)



3-AZA-CHRYSENE

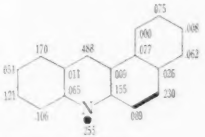


1'-AZA-3, 4-BENZPHENANTHRENE

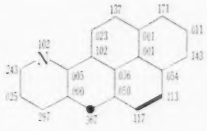


1, 2, 7, 8-DIBENZCARBAZOLE

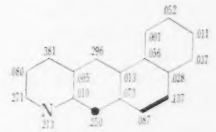
(C)



1, 2-BENZACRIDINE

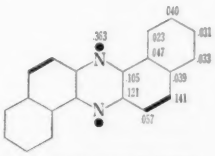


1'-AZA-3, 4-BENZPYRENE

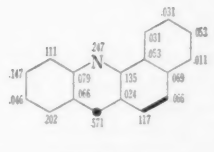


5-AZA-1, 2-BENZANTHRACENE

(C')



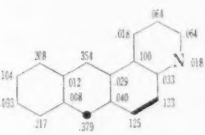
1, 2, 5, 6-DIBENZPHENAZINE



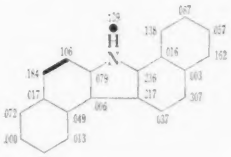
3, 4-BENZACRIDINE

Fig. 2. Frontier electron density (for electrophilic) and carcinogenophores. Thick line and black circle indicate the principal and the subsidiary carcinogenophores respectively.

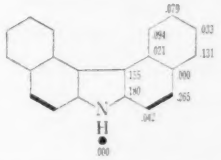
(A)



4'-AZA-1, 2-BENZANTHRACENE

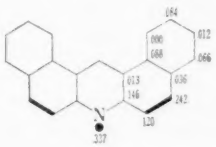


1, 2, 5, 6-DIBENZCARBAZOLE

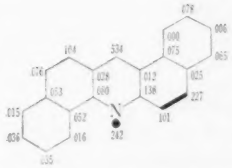


3, 4, 5, 6-DIBENZCARBAZOLE

(A)



3, 4, 5, 6-DIBENZACRIDINE



1, 2, 5, 6-DIBENZACRIDINE

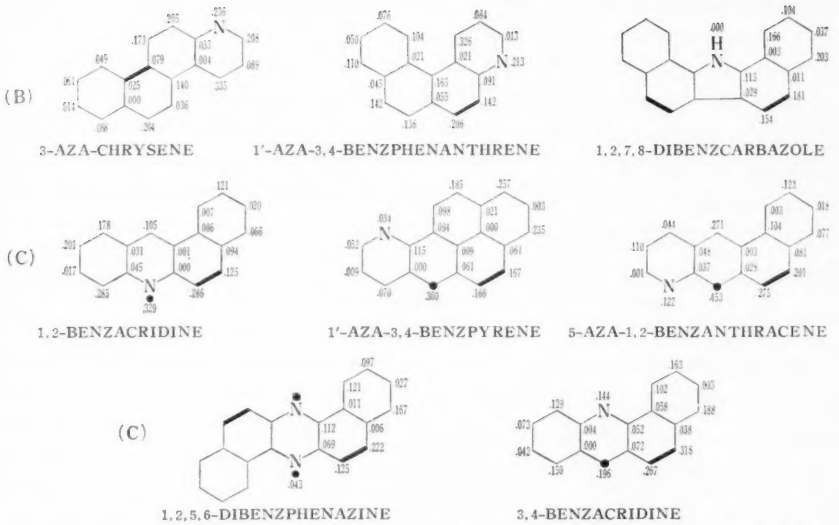


Fig. 3. Frontier electron density (for nucleophilic) and carcinogenophores. Thick line and black circle indicate the principal and the subsidiary carcinogenophores respectively.

Table 2. Relation between the frontier electron density at the principal carcinogenophore (f_p) of nitrogen-containing aromatic compounds and the experimental activity.

Group	Compound	f_p for nucleophilic**	f_p for electrophilic**	f_p for radical**	Carcinogenic activity
A	4'-aza-1,2-benzanthracene	0.480	0.258	0.369	±
	1,2,5,6-dibenzcarbazole	0.477	0.290	0.384	±
	3,4,5,6-dibenzcarbazole	0.413	0.307	0.360	+
	3,4,5,6-dibenzacridine	0.362	0.256	0.309	+
	1,2,5,6-dibenzacridine	0.328	0.495	0.411	+
B	3-aza-chrysene	0.378	0.523	0.450	−
	1'-aza-3,4-benzphenanthrene	0.349	0.399	0.374	−
	1,2,7,8-dibenzcarbazole	0.335	0.391	0.363	+
C	1,2-benzacridine	0.320	0.412	0.366	−
	1'-aza-3,4-benzpyrene	0.230	0.334	0.282	−
	5-aza-1,2-benzanthracene	0.224	0.475	0.350	−
	1,2,5,6-dibenzphenazine*	0.198	0.347	0.272	±
	3,4-benzacridine	0.183	0.585	0.384	−

* This compound has been reported experimentally inactive, but recent experiment indicated the tumor production by this compound when introduced into the bladder of young Wistar rats (44).

** The Coulomb integral of nitrogen atom was taken as $\alpha+\beta$ throughout the calculation.

subsidiary carcinogenophores are indicated by a thick line and black circle, respectively. In table 2, the values of frontier electron density at the principal carcinogenophore are listed with the experimental activity. At a glance and intimate correlation is seen between the values of frontier electron density for nucleophilic reaction at principal carcinogenophore and the carcinogenic activity. Especially, the above stated fact that 3, 4-benzpyrene loses its potent activity when the carbon atom at 1′ position is replaced by nitrogen atom was clearly explained as is seen in Table 2. Furthermore, the fact that the activity of a series of 1, 2, 5, 6-dibenzanthracene decreases with nitrogen replacement at mezo position was in good conformity with our theoretical index. Thus, the values of the frontier electron density at principal carcinogenophore of 1, 2, 5, 6-dibenzanthracene, 1, 2, 5, 6-dibenzacridine and 1, 2, 5, 6-dibenzphenazine become smaller with decreasing activity. The values of frontier electron density at subsidiary carcinogenophore were not included in the table, because they have shown no correlation with carcinogenic activity.

c) Azo compounds; 4-dimethylaminoazobenzene derivatives and related compounds.

4-Dimethylaminoazobenzene (DAB) and its derivatives are of considerable interest and have been regarded as a main group of carcinogens, because these compounds produce a high percentage of liver tumor in rats. However, not so many attempts have been made to correlate the carcinogenic activity of these compounds with their electronic structure.

Pullmans have suggested that the activity of these compounds is associated with an optimum range of electron density at the azo-linkage. In other words, they postulated that the carcinogenic reaction is electrophilic in nature (12, 26). Contrary to the Pullmans' theory the present authors have insisted that the carcinogenic action of these compounds will be originated from an interaction with nucleophilic center in the body (7). In that report we designated the dimethylamino nitrogen and the azo linkage as principal and subsidiary carcinogenophore, respectively. Thereafter, we have noticed the strong resemblance between the dimethylaminoazobenzene and polycondensed aromatic hydrocarbon with regard to their steric circumstances as is shown in Fig. 4 (9). In the figure, only the *trans* form of DAB was given, because this compound is found to take *trans* form predominantly (27). Furthermore, in connection with Fig. 4, the fact that 4-dimethylaminostil-

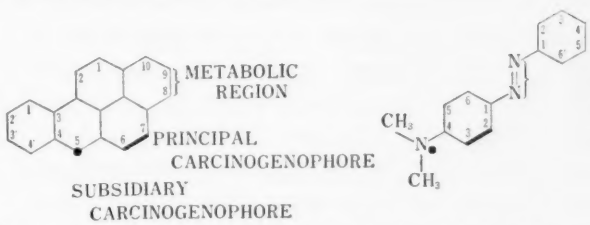


Fig. 4. Analogy between polycondensed aromatic hydrocarbons and DAB derivatives with regard to their steric circumstance.

bene which is found to be carcinogenic as well as carcinostatic is active only when it takes *trans* form and inactive in *cis* form is of special interest (28). As is seen in Fig. 4 we considered the azo linkage as a metabolic region of DAB and its derivatives. This might be permitted undoubtedly in view of the accumulated experiments (29). But the following remark must not be disregarded. The region concerned with the metabolism of aromatic hydrocarbons and DAB derivatives is not limited to the position marked in Fig 4. For example, the mezo region of 1, 2, 5, 6-dibenzanthracene has been known as the position of detoxicating cleavage (30, 31), and, with regard to DAB derivatives, different three pathways of metabolism have been found by experiment (29). That is, the reductive cleavage of azo linkage, the hydroxylation on the benzene ring and the oxidative demethylation of dimethylamino group.

The metabolic pathways stated above may be, in general, detoxicating one considering the fact that almost all of the metabolic products have no carcinogenic activity (21, 29). In the metabolism of DAB derivatives, reductive cleavage of azo linkage and hydroxylation on benzene ring were deemed to be detoxicating one (29), whereas oxidative demethylation proved to be intimately connected with the formation of bound dyes (32). Also attention must be paid to the fact that polycondensed aromatic hydrocarbons produce skin tumor, whereas DAB and its derivatives liver tumor only, and as will be stated later, designation of the principal carcinogenophore and the role of subsidiary carcinogenophore are not entirely the same as the case of condensed aromatic hydrocarbons.

With all things stated above, the fact that the carcinogenophores and metabolic region lie in a similar situation in both cases is of no little interest. Considering this analogy in sterical situation, we recognized the importance of positions 2 and 3 and calculated the frontier electron distribution at these positions in order to find a correlation between the theoretical indexes and experimental activity. The indexes at position 2, position 3 or both of these positions were compared with experiments, and the approximate superdelocalizability at position 2 for nucleophilic reaction proved to be the best index. This result is very interesting in view of the following experiment; Miller *et al.* (33, 34) and Nye (35) have examined the structure of bound dye and concluded that the binding would take place at position 2. As was already pointed out in our previous paper (7), however, a good parallelism was also found between the approximate superdelocalizability for nucleophilic reaction at dimethylamino nitrogen and the experimental activity. Hence in the case of DAB and related compounds, the role of aminoazo nitrogen is considered to be more important than that of the subsidiary carcinogenophore of polycondensed aromatic hydrocarbons and it may contribute significantly to the complex formation between the carcinogen and receptor in the body. In this sense, the terms " principal " and " subsidiary " seem inadequate

for this series of compounds.

The theoretical indexes were compared with the experimental activity in Table 3.

Table 3. Comparison between the theoretical indexes and carcinogenic activity of DAB derivatives and related compounds.

Compound	$S'^{(N)*}$ at 2 position	$S'^{(N)*}$ at dimethyl-amino nitrogen	Total π electron density at dimethylamino azo nigrogens	Carcinogenic activity
3-methyl-DAB	0.939	2.656	1.384	±
2, 6-difluoro-DAB	(0.914)	3.244	1.404	—
2′-fluoro-DAB	0.857	2.825	1.415	###
4′-fluoro-DAB	0.855	2.824	1.416	####
2-fluoro-DAB	0.843	2.893	1.416	####
3′-methyl-DAB	0.818	2.584	1.428	####
3′, 5′-dimethyl-DAB	0.818	2.584	1.428	—
3′-hydroxy-DAB	0.818	2.584	1.428	—
3′-fluoro-DAB	0.817	2.582	1.428	####
3′, 5′-difluoro-DAB	0.817	2.583	1.428	####
DAB	0.817	2.583	1.428	###
3′-chloro-DAB	0.817	2.583	1.428	##
2′-chloro-DAB	0.817	2.603	1.429	+
4′-chloro-DAB	0.817	2.602	1.429	+
2-methyl-DAB	0.751	1.920	1.471	—
2-hydroxy-DAB	0.749	1.950	1.471	—
4′-amino-DAB	0.734	2.210	1.455	—
4′-hydroxy-DAB	0.713	2.037	1.466	—
2′-hydroxy-DAB	0.711	2.036	1.468	—
4′-methyl-DAB	0.709	2.008	1.466	±
2′-methyl-DAB	0.701	1.989	1.469	+
4′-acetylamino-DAB	0.621	1.606	1.505	—
3, 5-dimethyl-DAB	0.477	2.705	1.340	+
p-dimethylamino benzal-aniline	0.427	0.418	1.702	—
N, N-dimethyl N-benzal-p-phenylene-diamine	0.209	0.243	1.761	—

* S′ is the approximate superdelocalizability and superscript N denotes nucleophilic.

The values of Coulomb and resonance integrals used in the calculation were indicated in Table 4. Coulomb integrals of methyl at dimethylamino group and of three nitrogen atoms were taken as $\alpha+2\beta$ and $\alpha+\beta$ respectively. α_X and α_{C_1} are the Coulomb integrals of substituent X and the carbon atom to which the substituent attached, respectively. β_{C_1-X} is the resonance integral between the substituent and carbon atom C_1. The compounds in Table 3 were arranged in the order of the value of approximate superdelocalizability at 2 position. 2, 6-Difluoro DAB was found to be inactive, while

Table 4. The values of Coulomb and resonance integrals used in the calculation of DAB derivatives.

Substituent, X.	α_X	α_{C_1}	β_{C_1-X}
CH_3	$\alpha+2\beta$	α	β
F	$\alpha+3\beta$	$\alpha+0.5\beta$	β
Cl	$\alpha+2\beta$	$\alpha+0.5\beta$	β
OH	α	α	0.7β
NH_2	$\alpha+0.46\beta$	α	0.4β
$NHCOCH_3$	$\alpha+\beta$	α	β

the theoretical indexes are the largest among these compounds. This is probably due to the fact that the binding position is wholly blocked by two fluorine atoms. 3-Methyl-DAB, 3′-hydroxy-DAB, and 3′, 5′-dimethyl-DAB, seem to be exceptions to our theory, because in spite of their large values of index, they are all experimentally inactive. To obviate this inconsistency, some other factors than the electronic structure should be taken into consideration.

Furthermore, by using the perturbation theory (46) we calculated the total π-electron density at aminoazo nitrogen of these compounds and the result was listed in the fourth column of Table 3. Comparing these values with experiment we can see a tendency that the larger the value is, the less carcinogenic the compound is. It may be no need to say that such a relation is a reflection of the nucleophilic nature of the carcinogenic reaction at the dimethylamino nitrogen.

The values of the theoretical indexes for electrophilic reaction at 3 position are of interest, because this position is considered to be very reactive toward an electrophilic reagent and Terayama *et al.* from their experiments postulated the binding at this position with protein residues (36, 37). As is seen in Table 5 the theoretical indexes at position 3 for electrophilic reaction are very large, therefore, the reaction at this position is easily expected. At the present stage of knowledge, it is difficult to decide which position, 2 or 3, is truely concerned with the binding in the process of carcinogenesis.

Haddow pointed out the necessity of free 4′ position of 4-aminostilbene derivatives for the occurence of the biological activity such as carcinogenic and anti-tumor activity (28). For DAB derivatives, the tendency is seen that the blocking at 4′ position makes the compound less active. Therefore, this position seems to have some concern with the biological activity. In this connection, the following fact may be worthy of an attention. That is, some of the present authors have been undertaking to accumulate the knowledge about the chemical reactivity of azo compounds with a view to correlate the chemical reactivity with biological activity. In our experiment, Michler's hydrol which is known as electrophilic reagent attacks 4′ position of DAB predominantly. This is true for other compounds such as 2′-methyl-

Table 5. Theoretical indexes for electrophilic, radical and nucleophilic reaction of DAB.

Position	$f^{(E)}$*	$f^{(R)}$	$f^{(N)}$	$S^{(E)}$*	$S^{(R)}$	$S^{(N)}$	Total π electron density**
2	0.011	0.062	0.112	0.876	1.209	1.542	0.982
3	0.154	0.116	0.077	1.275	1.275	1.274	1.028
2′	0.097	0.090	0.082	1.147	1.230	1.313	1.023
3′	0.002	0.001	0.000	0.826	0.826	0.827	0.998
4′	0.104	0.094	0.083	1.115	1.198	1.281	1.015
α-Nitrogen	0.093	0.291	0.489	1.067	2.400	3.733	1.286
β-Nitrogen	0.340	0.332	0.318	1.849	2.182	2.515	1.425
Dimethylamino nitrogen	0.616	0.485	0.353	2.734	2.733	2.732	1.428
Methyl group	0.082	0.059	0.036	0.937	0.603	0.270	1.953

* f and S are the frontier electron density and superdelocalizability respectively, and the superscripts E, R and N denote electrophilic, radical and nucleophilic, respectively. The larger these values are, the more reactive that positions are.

** Total π electron density has by other authors been used as the theoretical index discussing the chemical reactivity. The larger their value is, the easier the electrophilic attack is. For the nucleophilic attack, the reverse relation holds, that is, the smaller this value is, the easier the attack is.

DAB, 3′-methyl-DAB and MAB (monomethylaminoazobenzene). On the other hand 4′-methyl-DAB was entirely inactive owing probably to the blocking of the position of attack, and also 4′ position of azobenzene was found to be wholly inactive (38).

In conclusion, the discussion on the nature of the carcinogenic reaction is needed. Whether the reaction is electrophilic, radical, or nucleophilic is very important in connection with the knowledge of the site of reaction in the body. Pullman and other researchers have insisted the electrophilic nature of the carcinogenic reaction of condensed aromatic hydrocarbons, but recently Heller and Pullman (18) postulated that the carcinogenic reaction of DAB and its derivatives is nucleophilic. These two suppositions are contradictory to each other. Rather it may be inferred that the type of carcinogenic reaction of polycondensed aromatic hydrocarbon and their derivatives also might be nucleophilic in nature.

The present authors have iterated that the type of reaction of all chemical carcinogens may be nucleophilic in nature (7, 8, 9, 45). In support of this postulation the following facts may be pointed out: 1) significant degree of correlation was found between the frontier electron distributions for nucleophilic reaction at principal carcinogenophore of substituted aromatic hydrocarbons, nitrogen-containing heteroaromatic compounds and azo compounds and the experimental activity; 2) a series of nitrogen mustard, epoxides and other alkylating agents which are carcinogenic as well as carcinostatic, have been found to react easily with nucleophilic groups under physiological conditions; 3) carcinogenic metals, such as zinc, nickel, and beryllium, are

considered to interact as a cation with nucleophilic center in the body; 4) 4-nitro quinoline-N-oxide and its derivatives, whose potent carcinogenicity has been found by Nakahara et al. (39), proved to be very reactive toward a nucleophilic reagent such as sulfhydryl group in a test tube (40, 41) and Nakahara and Fukuoka assumed that the nucleophilic replacement at position 4 of this compound might be causally significant to the carcinogenesis (42); 5) the mechanism of plant hormone action has been expected to have some concern with the carcinogenic mechanism, because both of these compounds are similar to each other in bringing about the extraordinary cell division. As was pointed out by the present authors (43, 45), a strong resemblance was found between the plant growth compounds such as benzoic acid derivatives and carcinogenic compounds with regard to the steric circumstance and the type of reaction.

As the nucleophilic groups which may exist under physiological condition in the body, the followings were pointed out by Ross; that is, ionized acid groups such as $RCOO^-$, and $\equiv PO^-$, ionized form of hydroxy compounds, ionized sulfhydryl groups, amines and thioethers (11). There remain questions to be solved in the future which of these groups is truely connected with the occurrence of malignant growth, and which is the receptor for carcinogen, the protein or nucleic acid, and also what chemical group in them.

Summary

The electronic structure of methyl-substituted 1, 2-benzanthracenes, heteroaromatic compounds such as benzcarbazoles, benzacridines and DAB derivatives was calculated by the frontier electron method. A distinct parallelism was found between the frontier electron distribution at the carcinogenophore of these compounds and their carcinogenic activity, Reaction involved in the early stage of carcinogenic action of all chemical carcinogens has been postulated by the present authors to be of nucleophilic nature, and the numerical values of the reactivity indexes in this paper supprt this postulation.

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INDUCTION OF PULMONARY TUMORS IN MICE WITH ISONICOTINIC ACID HYDRAZID*

(Plates II and III)

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In the previous communication it was shown that a marked increase in incidence of lung tumors in dd mice followed the oral administration of isonicotinic acid hydrazid (INH) (1). When animals were fed with diet containing 0.3 per cent of INH, pulmonary tumors were induced as early as four months after the beginning of the feeding. And all animals sacrificed after seven months showed multiple (1 to 5) pulmonary tumors. Grossly and microscopically, these tumors were the same as those described by other investigators (2-8). The purpose of the present work was to determine in more precise manner the minimum dose of INH and other similar compounds required to induce pulmonary tumors.

EXPERIMENTS

1. Test for feeding of INH.

The experimental procedure was almost similar to that described in the previous paper. Strain dd female mice, $1\frac{1}{2}$ to 2 months old, were used exclusively. Mice were fed on the mixture of Oriental diet M and polished rice (1 : 2) containing varying amounts of INH. The dosage levels of INH added to the diet were ranged from 0.25 to 0.01 per cent of the diet. Of seven groups of fifteen to twenty animals each, five groups were fed with the diets of varying levels of INH, the remaining two groups, fed on the INH free diet, serving as controls. Green vegetables were given to the mice from time to time and water freely.

After seven months from the beginning of the feeding, all mice were sacrificed and examined for the presence of peripheral lung tumors. At autopsy, the lungs were removed and dropped into formalin solution. This causes the surface tumors to stand out as white elevated nodules against the normal lung tissue. The nodules measurring up to 1 to 3mm in diameter were counted with the unaided eye, and the number (peripheral index) was recorded. All doubtfull cases were examined microscopically. The peripheral index served for the purpose of comparison. It may be emphasized that the percentage of pulmonary adenomas reported may be a minimum number, because very small nodules might have been overlooked.

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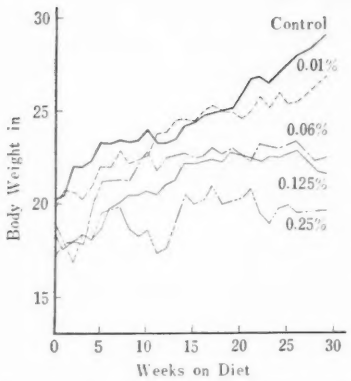


Fig. 1. Weight curves of mice fed various amounts of INH in the diet compared with that of controls.

Both experimental and control groups were weighed weekly. In the early period, those receiving the INH more than 0.06 per cent in diet began to lose weight and continued to do so for 1 to 3 weeks. After that their weight increased but throughout the period of observation it did not reach that of the controls (Fig. 1). Animals fed on the diet of 0.01 per cent grew at a normal or nearly normal rate. Microscopically, the tumor cells were cuboidal or columar, nuclei located in about the middle of the cells. The mitotic figures were not numerous. The cells were, in general, arranged in a single layer, and sometimes formed the glandular lumina in a papillary fashion or were relatively solid in appearance (Plates II and III). The stroma was scanty and consisted of a few fibrocytes. The tumors grew expansively. These growths were identical in structure with those described as spontaneous primary tumors of the lung, or induced tumors by carcinogenic hydrocarbons, urethane or nitrogen mustard by many previous investigators. Our induced pulmonary tumors in dd mice are in every way identical with the pulmonary tumors of spontaneous origin except for thier frequency and multiplicity. They arise from the alveolar lining of the lung. The parallelism between the suceptibility to spontaneous occurrence and development of pulmonary tumors with carcinogenic hydrocarbons suggests that the hydrocarbons or other compounds are accelerators of some process inherent in the animals.

The results are summarized in Table 1. Tumors of the lungs were induced in all experimental groups of mice which received INH diet, and the nodules ranged 1 to 5 in number. There is an accurate correlation between the dose of INH and the incidence or the average number of induced pulmonary tumors. Thus, in group 1 on the 0.25 per cent INH diet, all animals had an average of 1.85 tumors; in group 2 on the 0.125 per cent INH, 70 per cent of the mice had an average of 1.7 tumors, and in groups 3 and 4 on the diet of 0.1 and 0.06 per cent INH, 60 and 50 per cent of the mice had 1.0 and 0.9 tumors, respectively. One out of 12 mice in group 5 on the 0.01 per cent INH diet showed lung nodules multicentrically. And the carcinogenic index is derived by multiplying the percentage of animals in which tumors develop by the average number of tumors per tumor bearing animal: the agreement between the dose and the index values is evident. No animal in group 6 (controls) had a pulmonary tumor. Only one mouse showed a single lung tumor in group 7 (control 2), showing that the normal incidence of such tumors in this strain of mice is less

Table 1. Incidence of pulmonary tumors in mice fed various amounts of INH.

Group	INH dose in diet (per cent)	No. of mice surviving 7 months	Mice showing given number of tumors per individual 0 1 2 3 4 5	Incidence (per cent)	Mean number of nodules per mouse	Carcinogenic index
1	0.25	7	0 3 2 2	100	1.85	185
2	0.125	10	3 2 2 2 1	70	1.7	119
3	0.1	10	4 2 4	60	1.0	60
4	0.06	10	5 1 4	50	0.9	45
5	0.01	12	11 1	8	0.33	2.6
6 (control 1)	0.0	8	8	0	0	0
7 (control 2)	0.0	36	35 1	3	0.03	0.1

than 3 per cent within 7 months of experimental period. Then the pulmonary tumor found in group 5 could be classified as spontaneous, not induced by INH feeding. It is possible, however, that more tumors would have seen in this group if an interval of more than 7 months had been allowed before autopsy, but the results may be taken to indicate that 0.01 per cent INH diet did not enhanced markedly the carcinogenic incidence within 7 months.

These results indicate that the experimental animals showed a graded increased incidence of tumors according to INH dosage. And 0.06 per cent INH in the diet may be sufficient to induce tumors in 50 per cent of mice within 7 months from the beginning of the INH feeding.

2. The localization of the induced tumors in the lung.

Next, the localization of total 51 lung nodules induced in 26 mice in the preceding experiment was investigated. The result is shown in Table 2. In the table, 14

Table 2. Localization of the induced tumors in the lungs.

Group	INH dose in diet (per cent)	No. of animals with lung tumors (7 months)	No. of lung nodules induced				
			Right lobules			Left lobules	
			Upper	Middle	Lower	Upper	Lower
1	0.25	7	2	3	4	0	4
2	0.125	6	3	1	3	1	2
3	0.1	7	0	6	2	7	0
4	0.06	5	2	3	2	1	1
5	0.01	1	1	1	0	0	1
Total		26	8	14	11	9	9
Percentage			15.7	27.4	21.6	17.6	17.6
			100.0%				

(27.4%) and 11 (21.6%) nodules were located in the middle and lower lobules respectively, of right lung. And 9 (17.6%) nodules were found in each of the upper and lower lobules of left lung. Remaining 8 (15.7%) nodules were found in the upper right lobule. The middle right lobules seemed to show the largest number of nodules.

3. Minimum period of feeding for tumor occurrence.

An experiment involving 60 mice was carried out in the same procedure as before for determining the minimum period of feeding which is necessary for the induction of tumors.

Four groups of mice were fed 0.25 per cent INH diet as same as in the above experiment. After 2, 3 or 4 months of INH feeding, each group of animals was placed on a basal diet without INH for additional months. And all animals were sacrificed at 7 months after the beginning of the experiment, and examined for the presence of grossly tumors visible. The result is shown in Table 3, in which data on control

Table 3. Pulmonary tumors appearing after different periods of feeding.

0.2% INH feeding periods (months)	No. of mice surviving 7 months	Mice showing given no. of tumors per individual 0	1	2	3	4	Incidence (per cent)	Mean no. of nodules per mouse	Carcinogenic index
0 (controls)	20	19	1				5	0.05	0.25
2	5	3		2			40	0.8	32.0
3	8	4	3		1		50	0.75	37.5
4	8	4	2	2			50	0.75	37.5
7	7	0	3	2	2		100	1.86	186

groups and 7 month feeding groups were cited from the preceding experiment. The tumor incidence in animals fed INH diet for 2 months was 40 per cent, and those of animals for 3 or 4 months were both 50 per cent. Only one out of 20 animals fed normal diet showed a single pulmonary tumor. All the animals fed INH diet for 7 months continuously had single or multiple pulmonary tumors. It is shown that feeding the INH diet for a period of 2 months is necessary to induce pulmonary tumors in mice.

4. Test for subcutaneous injection.

In this series the INH was injected; 0.1ml of 2.0 per cent aqueous solution was administered subcutaneously to each animal on the back at as nearly the same site as possible. All the animals weighed about 20g at the beginning of the experiment. The dose was not sufficient to induce convulsion. The injection was repeated every

Table 4. Incidence of pulmonary tumors in mice injected INH subcutaneously.

Group	No. of mice surviving 7 months	Mice showing given number of tumors per induvidual 0	1	2	3	Incidence (per cent)	Mean number of nodules per mouse	Carcinogenic index
8 (INH)	15	7	5	1	2	53	0.87	46.1
9 (control)	9	8	1			11.1	0.11	12·2

two days for 18 weeks. For control, animals received the same amount of physiological salt solution for same duration of time. The result is shown in Table 4. The number of animals survived were 15 in the INH group and 8 in controls, and the percentages of the incidence of tumor were 53 per cent in the INH group and 12.5 per cent in controls. Lung tumors, and apparently only lung tumors and no tumor at the site of injection, were obtained.

5. Test for other compounds.

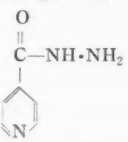
Preliminary to further study of the mechanism of action of INH, a number of other compounds have been studied with respect to carcinogenic activity for pulmonary tissue of the mouse. The compounds used in the experiment were pyrazinamide, sodium isonicotinyl hydrazine methansulfonate (Neoiscotin) and semicarbazide hyrochloride. Each compound was mixed well with basal diet. The food was given *ad libitum*. The animals gained weight on the diet. After seven months on the diet, all survivors were killed and autopsies made. As shown in Table 5, animals fed 3

Table 5. Incidence of pulmonary tumors in mice.

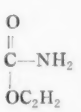
Group	Treatment	No. of mice surviving 7 months	Mice showing given no. of tumors per individual 0	1	2	3	4	Incidence (per cent)	Mean no. of nodules per mouse	Carcinogenic index
10	3% Pyrazinamide in diet	5	2	2	1			60	0.8	48
11	0.1% INH plus 20% Pyrazinamide in diet	12	5	4	2		1	58	1.0	58
12	0.4% IHMS* in diet	8	3	3	1		1	62.5	1.1	68.8
13	0.1% Semicarbazid in diet	8	2	4	2			75	1.0	75

* IHMS Sodium isonicotinyl hydrazine methansulfonate (Neoiscotin)

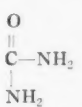
per cent of pyrazinamide in diet had 60 per cent incidence of tumors, with average of 0.8 nodules per mouse (Plate III). When animals received 0.1 per cent of INH



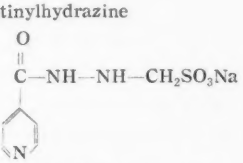
Isonicotinylhydrazine



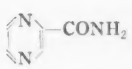
Urethane



Semicarbazid



Sodium isonicotinyl hydrazine methansulfonate (Neoiscotin)



Pyrazinamide

and 2 per cent pyrazinamide in diet, pulmonary tumors was induced in 58 per cent. And animals fed 0.4 per cent of Neoiscotin in diet for seven months had pulmonary tumors in 62.5 per cent. In this series of experiments, all compounds examined have relative potencies to induce pulmonary tumors in mice. There is remarkable similarrity in molecular structures among the compounds, all containing carbamyl group

$$(NH_2\text{--}\overset{\overset{O}{\|}}{C}\text{—}R).$$

Discussion

Since the discovery of INH, many attempt were made to elucidate the pharmacodynamic actions of the chemical. Recently, Juhász, Baló, and Kendrey reported that the carcinogenic action in mice was due to the intraperitoneal injections of the chemical (9). On the other hand, Nettleship and Henshaw reported the comparatively rapid induction of pulmonary tumors in mice with urethane (ethylcarbamate) (4). Subsequent work by Henshaw and Meyer showed that a single treatment produced some tumors, and the urethane could be administered in a variety of ways (intraperitoneal injection, subcutaneous implantation of crystals, or adding it to the drinking water) with the same result (10, 11).

This respose is similar to that to the carcinogenic hyrocarbons (2, 12) or to irradiation (13). We have had a little opportunity of testing tumor producing action of substances related to INH. The substances, which we did test, included pyrazinamide, Neoiscotin and semicarbazide hydrochloride. Among them former two are said also to be tuberculostatic and tuberculocidal *in vivo*, and latter one is of very similar structure. Feeding of these compounds as in INH experimets also induced pulmonary tumors in about 60 per cent of mice within 7 months. The similarity in molecular structures among urethane, INH, pyrazinamide, sodium isonicotinyl hydrazine methansulfonate (Neoiscotin) and semicarbazide hydrochloride consists in the presence of carbamyl group ($NH_2\text{—}\overset{\overset{O}{\|}}{C}\text{—}R$).

Summary

Isonicotinic acid hydrazide (INH), a tuberculostatic or tuberculocitic compound of wide use, induced pulmonary tumors when it was fed to mice with diet. Under the usual conditions of our experiments multiple pulmonary tumors were engendered in high proportions of animals surviving 7 months. Two months of feeding of INH are sufficient to induce pulmonary tumors in 60 per cent of animals whithin 7 months. The pulmonary tumors induced by INH feeding are apparently initiated by the hyperplasia of the alveolar epithelium and could be classified as adenomas identical

in appearance with the spontaneous pulmonary tumors that occur in the strain.

A few substances related to INH, namely pyrazinamide, Neoiscotin, and semicarbazide, also produce pulmonary tumors. All these compounds have carbamyl groups in their chemical structures.

We should like to thank Dr. K. Takeda, Shionogi Co., LtD., for gifts of chemicals.

Explantation of Plates II and III

Fig. 1. Gross appearance of tumor nodules in lungs of dd mouse fed INH diet.
Fig. 2. One area of pulmonary tumors in Fig. 1.
Fig. 3. Higher magnification of Fig. 2.
Fig. 4. Induced pulmonary tumors in a mouse with pyrazinamide.
Fig. 5. Higher magnification of Fig. 4.
Fig. 6. Silver stain of Fig. 5.

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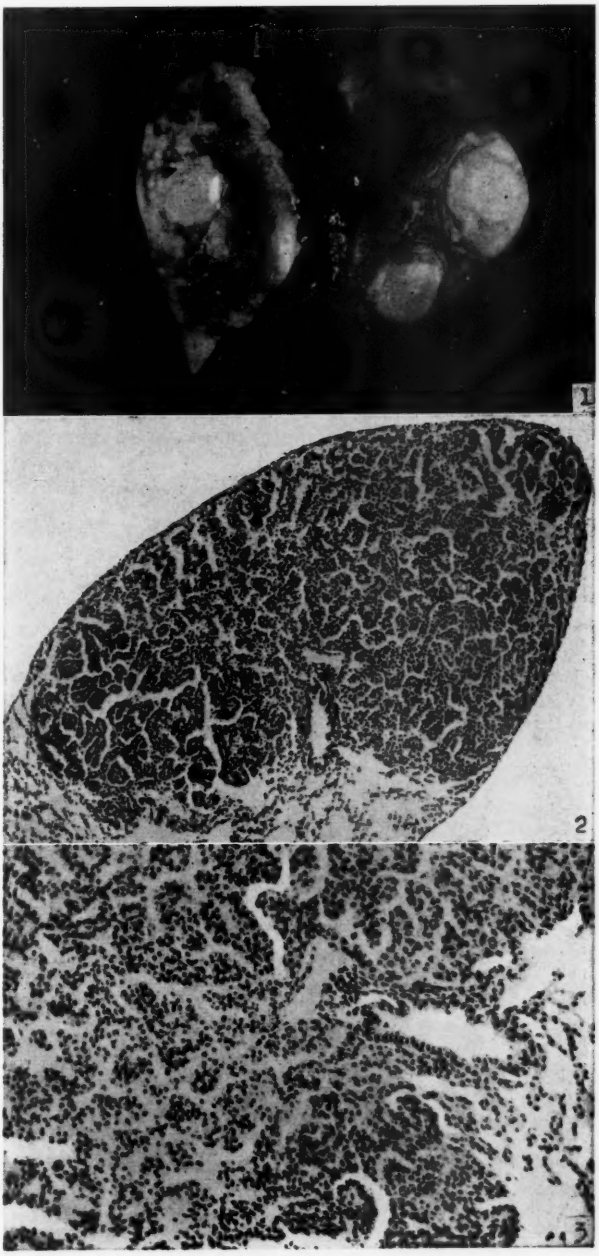
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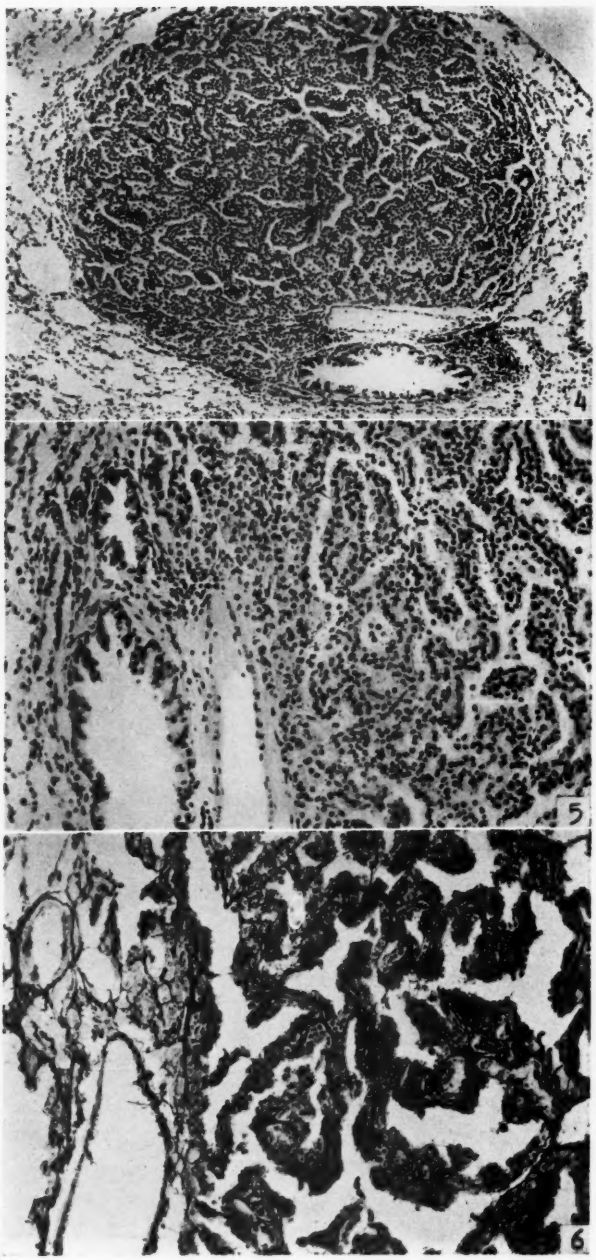
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[GANN, 51, 91~95; March, 1960]

EFFECT OF ISONICOTINIC ACID HYDRAZID ON HEPATOCARCINOGENESIS*

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Since the original description by Kinoshita (1) of the liver cancer resulting from the feeding of p-dimethylaminoazobenzene (DAB), numerous means of inhibiting the liver cancer production have been demonstrated (2, 3, 4). Investigations were made that the carcinogenic polycyclic hydrocarbons such as methylcholanthrene had a marked effect on hepatic carcinogenesis (5).

In our previous paper, a successful attempt was made to induce pulmonary tumors in mice maintained on a diet supplemented with isonicotinic acid hydrazid (INH) at a level of 0.3 per cent (6, 7). As lungs of mice are extremely susceptible to the induction of tumors with methylcholanthrene also, it has become of interest to determine whether or not the administration of INH would inhibit the production of liver cancer by the simultaneous feeding of azo-dye.

The results of this experiment are now reported since it became apparent that INH had a marked inhibitory effect on the development of the hepatic tumor and cirrhosis due to DAB.

EXPERIMENTS

Three groups of male albino rats (Wistar strain) were used for this experiment. The first group (control): 30 rats were maintained on the mixture of polished rice and Oriental diet M (3: 1) with 0.04 per cent of DAB dissolved in olive oil (DAB diet). The second group (0.1 per cent INH): 30 rats were kept on the same DAB diet to which INH was added at the level of 0.1 per cent. The third group (0.2 per cent INH): 20 rats were fed on the DAB diet containing 0.2 per cent INH. A slice of carrot was given thrice a week and water freely.

Records were kept on the average body weight and the average amount of food consumed during the experimental period (Fig. 1). The growth curve of rats receiving 0.1 per cent INH diet was similar to that of control. While a marked inhibition of growth was apprent in rats fed on 0.2 per cent INH diet, but no explantaion is available for this phenomenon at present. The average amounts of diets consumed per rat per day were 10g in the control, 9.8 g in the second and 9.5 g in the third group. It is thought that the difference within this range in amount of food-intake will not

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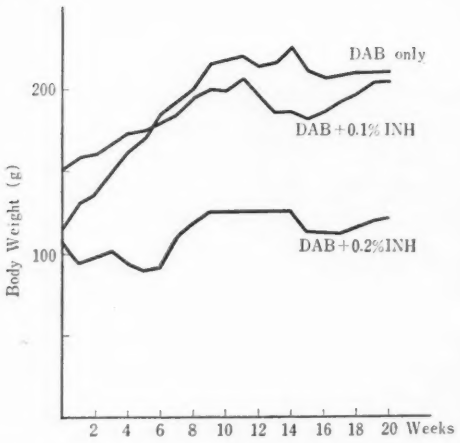

Fig. 1. The growth curves of rats fed various diets.

affect the degree of liver cancer production.

Some of the rats died early in the course of the experiment, too early to show any relevant change, and they were discarded. All the experiments were terminated at the 150th day after the commencement of experiments by sacrificing all the rats in order to make a final comparison of liver changes among the groups.

The results of the above experiments are summerized in Table 1, where the nature of liver fingings is classified as follows: liver cancer with marked annular cirrhosis, advanced or incipient cirrhosis and without cirrhosis.

The first group (control) included 15 cases (68.2%) of liver cancer accompanying annular cirrhosis, 5 cases (22.7%) of cirrhosis and the remaining 2 cases (9.1%) of liver without cirrhosis. In these two cases periportal fibrosis was slight and macroscopically livers were smooth. In the second group (0.1% INH diet), 17 (65.5%) of the total 26 rats showed apparently normal liver, in 4 cases (15.4%) the liver was cirrhotic and in the remaining 5 (19.2%) the hepatic changes were advanced to warrent

Table 1. Comparison of liver changes between the INH-diet fed and control dye-diet groups.

Experimental group	No. of experiment	No. of rats surviving 150 days	Liver of findings			
			Degree of cirrhosis			Cancer with cirrhosis
			Absent	Incipient	Advanced	
1st group Dye-diet only (control)	1	11	0	2	1	8
	2	11	2	0	2	7
		22	2	5		15
			9.1%	22.7%		68.2%
2nd group Dye-diet with 0.1% INH	1	10	7	0	1	2
	2	16	10	2	1	3
		26	17	4		5
			65.5%	15.4%		19.2%
3rd group Dye-diet with 0.2% INH	1	13	11	0	2	0
			84.6%	15.4%		0

the diagnosis of cancer. The liver findings in the third group (0.2% INH diet) were as follows: 11 cases (84.6%) of liver without cirrhosis and the remaining 2 case (15.4%) with cirrhosis. No liver cancer was induced in the rats of this group. It is of special interest to note that these cirrhotic livers were accompanied with many cysts. Microscopically, there was a considerable proliferation of bile ducts with formation of clusters of ducts. Many of these bile ducts were dilated and formed cysts of various sizes. The lining cell of some cysts were greatly attenuated, but in other cysts they were cuboidal or columnar in shape. This type of cirrhosis was often encountered in rats fed on the flourene derivatives.

The results of the experiments described above clearly demonstrated that the INH at the level of 0.2 per cent in the dye diet has inhibited markedly the induction of liver cancer, but when the amount of INH was reduced to one-half the inhibiting effect was slightly less.

Discussion

INH has a marked effect in preventing the formation of liver tumor with DAB. The binding of amino azo-dyes to cellular constituents, probably protein, was suggested by Miller and Miller who extracted crude protein bound dyes (8). Terayama et al. considered that the polar dye was synthesized by Mannich reaction and they synthesized amino acid bound dyes by this reaction (9).

In our study, Mannich reaction between INH and either 4-monomethyl-aminoazobenzene (MAB)* or 4-aminoazobenzene (AB)* was investigated. An example of the reaction and the results are shown here:

AB (or MAB) alcohol solution (12×10^{-3}M), 5 ml, INH in 2 per cent $NaHCO_3$ solution (24×10^{-3}M), 5 ml, $NaHCO_3$ solution (2 per cent), 2 ml, and formalin solution (35 per cent), 2 ml, were mixed and incubated overnight at 35°C. From this reaction mixture were isolated: AB-INH polar dye 6.1 γ, and MAB-INH polar dye 3.8 γ (expressed as DAB).

These results suggested that INH bound azo-dyes as well as liver protein, and so inhibited the protein-binding of azo-dyes by combined itself with azo-dyes; it may be considered that a competition existed for azo-dyes between INH and protein. But this agrees with the suggestion of Baba that the inhibitory action of p-hydroxypropiophenone on the hepatoma induction with DAB may be due to the competition between these chemicals for liver protein (4).

The effect of INH on hepatic metabolism in rats fed DAB diet was examined. Two groups were prepared, in one of which rats were fed 0.04 per cent DAB diet for a month and in the other group rats fed 0.04 per cent DAB plus 0.2 per cent INH diet for a month. Polar dyes were extracted from the rat liver of the two groups

*We should like to thank Dr. Terayama for gifts of chemicals.

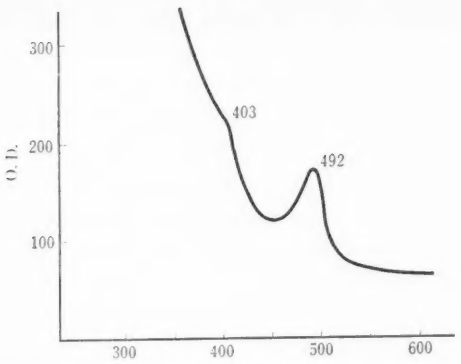

Fig. 2. Absorption curve of polar dye extracted from fresh liver.

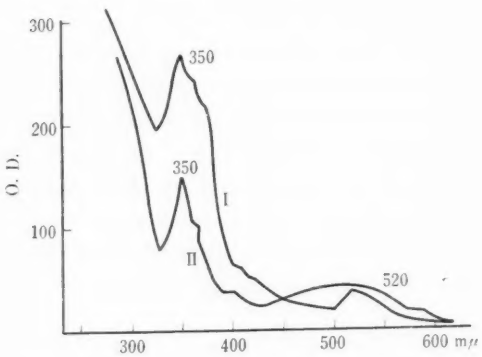

Fig. 3. Absorption curves of natural polar dye.
Curve no. I. Dye diet with 0.2% INH.
Curve no. II. Dye diet only.

by enzymatic hydrolysis method (10). After defatting liver by boiling it with methanol, the liver powdre obtained was extracted methanol. Spectrum of the polar dye so obtained is shown in Fig. 2. The spectral character of polar dyes from the two groups is quite the same. But they were different quantitatively, only, 2.3γ /10 g of fresh liver for DAB diet group, 5.6γ/10 g of fresh liver for DAB plus INH diet group (expressed as DAB). The extracts were then treated enzymatically, subjected to the alumina colum chromatography and the water eluates were collected. To the eluates were added 1 N HCl and the absorption spectra measured by Beckman spectrophotometer. Spectra of these polar dyes are shown in Fig. 3. There was no remarkable differencies between DAB only diet group and DAB plus INH diet group. Only the optical density of absorption maximum at 350 mμ differentiated them. The evidence of competition between INH and liver protein for azo-dye was not shown to exist, but the Mannich reaction between INH and polar dye may exist.

Gelboin et al. reported that *in vitro* a type of protein bound dye was formed when fortified with reduced pyridine nucleotides and incubated aerobically with either 3′-methyl-MAB or 3′-methyl-AB (11). It was reported by Zatman et al. that INH could replace the nicotinamide moiety of DPN by the action of enzyme, namely, INH-insensitive DPNase, and then become inactive as DPN (12). The possibilities that DAB metabolism is affected by INH is now under investigation in this laboratory.

Summary

When INH was added at the level of 0.1 or 0.2 per cent to 0.04 per cent DAB

diet, the liver cancer production due to DAB at the 150th day was markedly inhibited. The possible inhibitory mechanism of INH for azo-carcinogenesis was discussed but leaves much to be investigated in the future.

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CHEMICAL ANALYSIS OF CIGARETTE TAR PRODUCED BY HUMAN SMOKING

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INTRODUCTION

During recent years a number of papers have indicated several kinds of carcinogenic polynuclear hydrocarbons to be present in cigarette tar. These include 3, 4-benzpyrene,[1—3),5),7),13),14),17),20] 1, 2-benzpyrene,[1),12),15),16),17] 1, 2-benzanthracene,[9),12] 1, 2 : 5, 6-dibenzathracene[17] and 3, 4 : 9, 10-dibenzpyrene. In order to collect a large starting material for the analysis, use of one of the varieties of smoking machine has been the rule. They were usuallly designed to conform as nearly as possible to human smoking. It is known, however, that the way of smoking cigarettes, especially the pressure and duration of a pull, is subject to remarkable variation from person to person, and there may be even national and racial difference. More relevant data are expected if suitable material can be collected from the usual human smoking.

The present study was attempted to obtain data concerning the appearance of possible carcinogens based on the material smoked by a number of laboratory collaborators which may represent average smoking ways of the Japanese. Lindsey and Cooper[7] reported data obtained from an analysis of silica gel filters, but they were apparently used inserted in the ductlets of a smoking machine.

MATERIAL

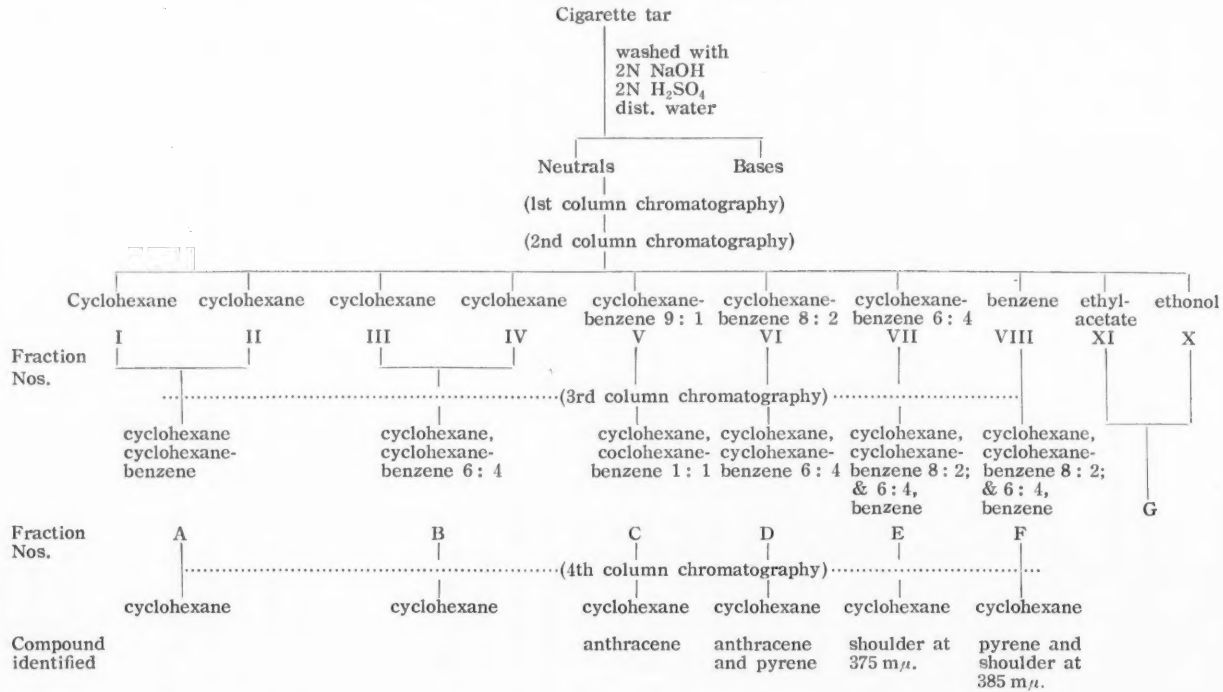
The cigarette tar used in this study was collected from a total of 3,500 cigarettes of popular Japanese brand "PEACE", smoked in the usual way by 12 of our laboratory collaborators through glass wool filter held in a special glass cigarette holder. Each filter was used for about 10 cigarettes. The tar on the filter was removed by extraction with benzene in a Soxhlet apparatus for 48 hours, the solvent evaporated off in vacuo at 50 to 60°C and residue dissolved in 20 ml of benzene.

FRACTIONATION

The benzene solution above was washed in turn with 2N sulphuric acid (3 times), 2N sodium hydroxide and distilled water, each 3 times. To this neutral fraction was added the some quantity of unhydrous sodium sulfate.

The chromatographic fractionation of this sample was carried out in the following

Table 1. Fractionation of cigarette tar.



four steps (Table 1). First the dark brown neutral fraction was passed through a large chromatographic column of aluminium oxide (Merck) (2.8×13 cm). Then the column was eluted with 1,400 ml of cyclohexane-benzene mixture (6 : 4) and 500 ml of benzene. Most of the strongly absorbable material was held as a dark brown zone at the top part of the column, while other substances were spread out on the lower half zone of the column. When the eluting solvent had passed through the column, ethylacetate was next run through, washing out a great deal of the yellow colored material in this step. Finally the column was washed through with ethanol, which yielded a dark orange-brown fraction. This material was kept as a separate fraction. The column was then discarded although some material was still retained on it.

All the eluates were combined and evaporated to dryness and residue dissolved in 5ml of pure cyclohexane. This solution was chromatographed on aluminum oxide column, 2.8×30 cm, eluting successively with 1,400 ml of cyclohexane, 200 ml of cyclohexane-benzene (9 : 1), 200 ml of cyclohexane-benzene (8 : 2), 200 ml of cyclohexane-benzene (6 : 4), 200 ml of benzene and 200ml of ethylacetate, finally with 100 ml of ethanol. The volumes of the eluates obtained after the 2nd column chromatography are shown in Table 2.

Table 2. 2nd column chromatography.

Fraction	Eluents	Volume of eluate from each fraction (ml)
I	cyclohexane	350
II	cyclohexane	350
III	cyclohexane	350
IV	cyclohexane	350
V	cyclohexane-benzene 9 : 1	200
VI	cyclohexane-benzene 8 : 2	200
VII	cyclohexane-benzene 6 : 4	200
VIII	benzene	200
IX	ethylacetate	200
X	ethanol	200

Fractions I and II in the 2nd column chromatography were combined, the solvent evaporated off in vacuo at 50 to 60°C (A) and residue dissolved in 2 ml of cyclohexane. Similary, fractions III and IV were combined and evaporated in the same condition (B). The fractions V, VI, VII and VIII were separately evaporated in similar method (C, D, E and F respectively).

In the 3rd column chromatography, A, B, C, D, E and F were chromatographed a third time using the same type of alumina column (1.5×15 cm) as before.

The eluates of A and B showed nothing.

C was eluted with 100ml of cyclohexane and then with 150ml of cyclohexane-benzene

solution (1 : 1). The first 120 ml and the last 115 ml of eluate showed nothing. Only the middle 15 ml gave an absorption peak or a shoulder at 375.1mμ. This fraction was evaporated to dryness and dissolved in 2ml of cyclohexane, chromatographed on silica gel (Mallinckrodt Chemical Work.), 1.5×3 cm, and eluted with pure cyclohexane. The first 9 ml of eluate showed nothing, but next 18 ml gave an absorption peak at 374.8 mμ probably due to anthracene.

Eluates of the D showed 2 absorption peaks, namely at 375 and 335 mμ. These peaks suggested that anthracene and pyrene are present in these samples. The eluates showing a peak at 375 mμ were combined, solvent evaporated off, and residue dissolved in 2ml of cyclohexane. This solution was chromatographed on silica gel, 1.5×3 cm, eluting with pure cyclohexane. First 10ml of eluate showed nothing. The next 20 to 50 ml gave an absorption peak at 375 mμ probably due to anthracene. The other eluate fractions of the D, showing absorption peak at 335 mμ, were similarly chromatographed on silica gel, and eluted with cyclohexane, this eluate showed a peak at 335 mμ, probably pyrene.

The eluates and their quantities are shown in Table 3. The eluates of E and F

Table 3. 3rd column chromatography.

Fraction	Eluents	Volume of eluate from each fraction (ml)
A	cyclohexane	150
	cyclohexane-benzene 1 : 1	150
B	cyclohexane	150
	cyclohexane-benzene 6 : 4	150
C	cyclohexane	100
	chclohexane-benzene 1 : 1	150
D	cyclohexane	150
	cyclohexane-benzene 6 : 4	150
E	cyclohexane	50
	cyclohexane-benzene 8 : 2	50
	cyclohexane-benzene 6 : 4	50
	benzene	100
F	cyclohexane	100
	cyclohexane-benzene 8 : 2	100
	cyclohexane-benzene 6 : 4	100
	benzene	50

could be purified further by column chromatography but these were complex mixtures and showed intense back ground absorption.

The eluate of E showed small peaks at 375 and 347.2 mμ, but there were no definite peaks anywhere else.

F was eluted with 100 ml of cyclohexane, 100ml of cyclohexane-benzene (8 : 2), 100

ml of cyclohexane-benzene (6 : 4) and 50 ml of benzene. The ultraviolet absorption curves of these eluates were without definite peak but showed intense back ground absorption. These eluates were combined, evaporated, and residue dissolved in 1 cc of pure cyclohexane. This solution was chromatographed on silica gel, 1.5×10 cm, eluting with pure cyclohexane. The addition of cyclohexane started a yellow material to run down the column. When irrradiated with ultraviolet light, this yellow material fluoresced in bright blue. The first 20 ml of eluate showed nothing. The next 40 ml fluoresced and showed a prominent peak at 334.8mμ and a shoulder peak at 375 mμ. The next 50ml of it showed nothing, but the following 150ml had shoulder peaks at 315 mμ, 365 mμ, 383.6 mμ, 384.3 mμ and 385 mμ (Table 4).

Table 4. 4th column chromatography.

Fraction	Eluents	Tube numbers	Compound identifeid	Volume of eluate from each fraction
C	cyclohexane	4, 5, 6, 7, 8, 9	anthracene	50 ml
D	cyclohexane	3, 4, 5, 6, 7, 8, 9	anthracene and pyrene	50 ml
E	cyclohexane	from 16 to 30	shoulder peak at 375 mμ	50 ml
F	cyclohexane	5, 6, 7, 8, 9	pyrene and shoulder peak at 385 mμ	350 ml

Results

All the fractions were tested in cyclohexane with a Beckmann DK-2 type ratio recording spectrophotometer.

The substance appearing in the fractions D and F above showed definite peaks at 334.8 mμ, 318 mμ and 305.3 mμ. The peak at 305.3 mμ was obscured by back ground absorption due to impurity, but this substance was considered as consistent with pyrene.

The substances which showed up in the fractions C, D and F had peaks at 374.8 mμ, 356.0 mμ and 339.1 mμ, and could be regarded as consistent with anthracene.

Further purification for more exact identification of the substance was not attempted.

A rough quantitative estimation of pyrene and anthracene, using the method of Cooper[3] and Alvord[2], gave the amounts as 1.06μg/100 cigarettes and 3.06μg/100 cigarettes, respectively.

Pure 3, 4-benzpyrene gives in cyclohexane peaks at 385 mμ, 367.1 mμ and 347.4 mμ. Although the fraction F showed small shoulder peaks at 385 mμ and 365 mμ, further attempts to purify this fraction into a definite component which might give comparable absorption curve with that of 3, 4-benzpyrene, were completely futile. Fluorescence emission spectra also ended in a negative result.

Discussion

Although it is advisable to start with a large amount of tabacco tar produced by means of smoking machine, if every minor component of the tar is to be detected, a test for detection of some important hydrocarbons from the cigarette smoke smoked in a physiological condition should have a value of its own.

After several series of experiments on the tars obtained by burning a few Japanese brands of cigarette, the author tried to analyse cigarette tar collected on glass wool in glass holders which were used for smoking by the laboratory members. Four steps of column chromatography were applied to the neutral portion of the tar. Anthracene and pyrene were identified and their estimated quantities were calculated as 3.06μg and 1.06μg per 100 cigarettes.

According to the previous report[7], anthracene and pyrene amounted respectively to 10.78μg and to 11.21μg per 100 cigarettes when the whole tar was collected by means of smoking machine. Cooper and Lindsey[7] have reported their result of analysis made on tars adsorbed on silica gel filter (DENICOTEA). They evaluated the anthracene and pyrene to be present absorbed on the filter as 20.0μg and 22.0μg respectively per 500 cigarettes. Low value of the two substances in the present study is quite understandable inasmuch as only parts of the smoke is adsorbed on the filters.

3 : 4 benzpyrene, which has been considered by the majority of the previous authors as the most important of the cacrinogens in tobacco tar, failed to be identified in the present study. There may be several factors which should be taken in consideration as accounting for this failure.

First, the whole amount of tar material collected in the present study from 3,500 cigarettes may be too little in quantity for the method of analysis. Izawa[11] has pointed out that the glass holders with stuffed glass wool would not collect more than 20% of the whole tar produced. If this is true and if each of the components is adsorbed at the same rate, the present data should be multiplied five times to represent their contents in the whole tar. Even the values so multiplied, namely, 5μg/100 cigarettes for pyrene and 15μg/100 cigarettes for anthracene, are remarkably low as compared with those in the previous reports. A preliminary investigation to get informations as to the minimal values of 3, 4-benzpyrene detectable by the method used, indicated that it was 0.2μg in 2.0ml of solvent. If, 3, 4-benzpyrene exists in the tar at all at the rate of 1/10 of anthracene, as in the case of Cooper and Lindsey, the substance should have been indentified.

Possible explanation to the failure of detecting 3, 4-benzpyrene may be either that the human smoking yields different products in the pyrolysis, that the same products in different ratio are obtained by the smoking machine, or that the adsorption on the

filter was selective, so that the important hydrocarbon was missed partly or completely.

Summary

1. The cigarette tar was collected from a total of 3,500 cigarettes of popular Japanese brand PEACE, smoked in the usual way by the people through glass wool filter held in a special glass cigarette holders.

2. The tar has been extracted and the neutral extract in cyclohexane has been examined by chromatography followed by ultraviolet absorption spectrophotometry.

The amount of pyrene and anthracene were calculated as 1.06μg/100 cigarettes and 3.06μg/100 cigarettes respectively.

3. 3, 4-Benzpyrene was not detected in the tar.

4. Possible causes for the failure in detecting the important carcinogen and for low values obtained of other hydrocarbons are discussed.

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[GANN, 51, 105~112; March, 1960]

HISTOCHEMICAL AND AUTORADIOGRAPHIC STUDY OF EXPERIMENTAL EPIDERMAL TUMORS

(Plates IV—VII)

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INTRODUCTION

From biological point of view, the keratinization has been assumed to be a differentiation process of the epidermal and epidermoid tissue cells.[12] Recent autoradiographic studies,[4),5),16)] using radiosulfer-containg aminoacids, appear to strongly support this assumption. According to the studies, the keratinization is an active synthetic process of a fibrous protein in the keratinizing tissues, especially in the granular layer and keratogenous zone.

Following up these results, it may be possible to obtain a series of data which will correlate biochemical changes and morphological characteristics in the keratinizing tissues. Further, some pathological problems, concerning the epidermoid metaplasia and the epidermal or epidermoid tumor, may be partly solved along this line.

The present study aimed, through histochemical and autoradiographic examinations, at the analysis of keratinization in the methylcholanthrene induced epidermal tumors and hyperplastic epidermis in mice. Special attention was paid to find out some differences in the keratinization-pattern between those and normal tissues.

MATERIAL AND METHOD

40 male mice of dd-strin, initially weighing about 16g, were used. They were divided into two groups. In group I, 30 mice were painted 30 times with 0.02 ml of 0.6% solution of 20-methylcholanthrene in benzene on the interscapular region of the dorsal skin during the first 10 weeks. They were left 80 days thereafter without any treatment. In group II, 10 animals were painted 6 times for 17 days with the solution of methylcholanthrene during the first 2 weeks and left untreated thereafter. The hairs were pulled off over the treated area, 24 hours before the treatment.

The S^{35} labeled cystine was administered intraperitoneally in a dose of 1.5μc/g to 14 mice which survived in the group I and to the selected 6 mice of group II. They were sacrificed, 3, 8, 24 and 72 hours respectively after the injection of radiocystine,

according to the experimental design (Table 1).

The histochemical and autoradiographic examination were made on treated and untreated parts of the skin and also on esophagus and tongue of these 20 animals. Those tissue sections were excised fresh and fixed in 1% solution of trichloracetic

Table 1. Experimental groups.

Experimental Group	Experimental days	Mc-painting (times)	Dose ef cystine-S^{35} (ip, c/g)	Hours after injection of cystine-S^{35}	Nos. of mice
I	150	30	1.5	3	3
				8	7
				24	2
				72	2
II	30	6	1.5	3	2
				8	2
				24	1
				72	1

acid in 80% ethanol. The paraffine sections were cut at 6μ.

Stripping method was applied for autoradiography. The slides were exposed for 4 weeks at 4°C and developed.

Barrnett-Seligman's DDD method[1),2),10)] was used for the histochemical detection of protein-bound sulfhydryl groups.

Results

A. Pathological findings of the skin lesions (Table 2):

1. Group I: Of the 14 mice, 12 had epidermal tumors, ranging between millium to bean in size, and grossly appearing papillomatous, and 4 of them were ulcerated at the surface. Histological examination revealed 5 squamous cell carcinomas, 6 papillomas and 1 pseudo-epitheliomatous hyperplasia. One of the squamous cell carcinomas closely resembled histologically the human keratoacanthoma, showing a central crater filled with keratin. Another carcinoma simulated basal cell carcinoma of the human skin. The diagnosis of the malignancy was based on the increased rate of the growth, morphological atypism of the cells and the invasion of the dermis. No distant metastasis were observed in any case.

The epidermis, surrounding the tumor, was as a rule remarkably thickened and hyper-and/or para-keratotic. The acanthosis was often associated with slight papillary outward proliferation and elongation of the rete pegs downwards.

Mast cell infiltration, elastosis and fibrosis in the dermis were observed more or less in all cases. Epidermal cysts were noticed in 9 cases.

2. Group II: No tumor appeared. The epidermis of the treated areas showed a prominent acanthosis with hyper- and/or para-keratosis. The appendages, including

Table 2. Pathological findings of the skin lesions.

Group		Tumor		Epidermis						Dermis		
Findings		Gross	Histology	Acanthosis	Basal cell proliferation	Hyperkeratosis	Parakeratosis	Horn pearl	Epidermal cyst	Mast cell	Fibrosis	Elastosis
I	1	Miliary Ulcerated	Papilloma	++	+	++	+	+	−	++	+	+
	2	Bean-sized Ulcerated	Carcinoma	++	+	++	+	+	++	+	−	++
	3	Miliary	Papilloma	+	−	+	+	+	+	++	+	+
	4	Rice-sized Ulcerated	Carcinoma	+	+	+	+	−	+	+	++	++
	5	Miliary	Papilloma	+	+	++	+	−	++	+	+	−
	6	Azuki-sized Ulcerated	Papilloma	++	+	++	+	+	++	++	+	+
	7	Miliary	Papilloma	+	+	+	+	−	+	+	+	+
	8	—		++	++	+	+	+	−	+	++	+
	9	—		+	++	++	+	+	+	++	++	+
	10	Azuki-sized	Carcinoma	++	+	++	+	+	−	+	+	+
	11	Bean-sized	Papilloma	++	+	++	+	+	−	+	+	+
	12	Rice-sized	Pseudo-epitheliomatous hyperplasia	+	−	++	+	+	−	+	+	+
	13	Bean-sized Ulcerated	Carcinoma	+	+	++	+	+	+	++	+	++
	14	Rice-sized	Carcinoma	++	+	++	+	+	+	++	+	+
II	1	—		++	−	+	+	−	++	−	+	−
	2	—		+	−	+	+	−	+	−	−	−
	3	—		++	−	+	+	−	−	−	−	−
	4	—		++	−	+	−	−	−	−	−	−
	5	—		+	−	+	+	−	+	−	−	+
	6	—		++	+	++	+	+	++	+	−	+

hair-follicles and sebaceous glands, were partly atrophied, destroyed or diminished. In some cases, the hair-follicles represented various stages of the cycle in one tissue-section.

Moderate edema, hyperemia and round cell infiltration were noticed in the dermis.

In either group, no remarkable lesions were observed in the tongue and esophagus.

B. Histochemical findings (Table 3):

1. Intact parts of the skin: A moderate and diffuse purple coloration occurred by the Barrnett-Seligman technique in the cytoplasma of the Malpighian layer cells. In some parts of the epidermis, surrounding the hair-pouch, an intense staining in blue was noticed. The cornified layer appeared faintly red. The histochemical reaction of the hairs and hair-follicles was intense in the suprabulbular regions,

Table 3. Comparison of the histochemical and autoradiographic findings.

Histology		Histochemical findings (-SH)	Autoradiographic findings (Intensity of blackening)			
			3 hours	8 hours	24 hours	72 hours
Normal epidermis	Malpighian layer	+～++	+	++	+	±
	Cornified layer	−～±	−	−	±～+	++
Tongue and Esophagus	Basal layer	+	+	+	+	±
	Spinous layer	++	+	++	+	+
	Granular layer	+++	+++	+++	+++	++
	Cornified layer	±～+	−	−	+	++
	Conical papilla (tongue)	+++	+++	+++	+++	+++
Hyper-plastic epidermis	Basal layer	+	+	+	+	±
	Spinous layer	++	+	++	+	+
	Granular layer	+++	+++	+++	+++	++
	Cornified layer	+++ or ±	−	±	++	++
Papilloma	Basal layer	+	+	++	+	±
	Spinous layer	++	++	++	+	+
	Granular layer	+++	+++	+++	+++	++
	Cornified layer	+++	−	±	++	++
Carcinoma	Undifferentiated	+	+	+	+	
	Differentiated	++	+	++	++	
	Terminal cells	+++	+++	+++	+++	
	Cornified layer	+++	−	+	++	

moderate in the bulbs and negative in the fully keratinized portions.

2. Esophagus and tongue: The granular layers of the tongues and esophagus mucosa were strongly stained in blue. The spinous and basal layers of these tissues showed a moderate and slight purple coloration respectively. The conical papillae of the tongue showed a high-SH content, appearing most intensely blue.

3. Hyperplastic epidermis: The basal cells of the hyperplastic epidermis were stained moderately in the nucleoli and slightly in the cytoplasma. The spinous layer was stained moderately in purple, the granular layer intensely in blue. The keratohyaline granules were negative in the reaction. The cornified layer showed a different staining-property, according to the histological types. Para- or hyperparakeratotic area in some cases showed a very intense staining in blue. However, the hyperkeratotic portions in the other cases showed only a slight staining.

4. Papilloma: Little difference in the histochemical reaction was noticed between the papilloma and hyperplastic epidermis. However, the most parts of the cornified layer or mass showed much staining in blue.

5. Carcinoma: The histochemical findings of the carcinomas were different from

case to case, corresponding to their morphological variability. However, a definite staining property was observed in the cytochemical order. The undifferentiated cells (basal cell type) were stained purple in the nucleoli and faint red in the cytoplasma. The differentiated cell (spinous cell type) were stained purple in the cytoplasma. The terminal cells (granular cell type) were stained in blue. The terminal cells (granular cell type) were stained in blue. The cornified portions, including horn-pearls and the mass in the central crater, were also stained in blue.

C. Autoradiographic findings (Table 3):

1. Intact parts of the epidermis: At 3 hours after the injection of radio-cystine, the Malpighian layers showed weak radioactivity, which became intensified at 8 hours. The cornified layers was not radioactive during the first 8 hours. It became active first at 24 hours.

The supra-bulbular regions of hair-follicles showed a very intense radioactivity already at 3 hours. In the fully keratinized parts of the hairs, no radioactivity was noticed within the first 8 hours. The lower portions of the keratinized hairs became radioactive at 24 hours.

2. Tongue and esophagus: The granular layers showed a very intense radioactivity 3 hours after the injection. The portion showing the most conspicuous radio-activity was conical papillae of the tongue. The basal and spinous layers showed slight and moderate activities respectively 8 hours after the injection.

3. Hyperplastic epidermis and papilloma: The incorporation of radio-cystine was most prominent in the granular layer, already 3 hours after the injection. The incorporation at the spinous and basal layers were slight and moderate respectively at 8 hours.

Although no incorporation of radio-cystine was noticed in the cornified layers within the first 8 hours after the injection, the bordering portion to the granular layer showed a moderate or intense activity at 24 hours.

4. Carcinoma: The uptake of radio-cystine injected was low in the undifferentiated cells (basal cell type), moderate in the differentiated cells (spinous cell type) and most prominent in the terminal cells (granular cell type). The radioactivity at the cornified layer or mass, including horn-perls and the mass in the central crater, were negative within the first 8 hours after the injection. However, in some parts, where marked proliferative processes were histologically observed, an intense radioactivity was noticed at the portions bordering the uncornified cell mass.

Little difference in the autoradiographic findings was noticed between the main mass of the carcinoma and the invading cell nests.

Discussion

A. Normal keratinization:

Histochemical studies of the keratinization have been reported by many authors.[2),3),6),13),9)] According to the previous reports, a quantitative histochemical distribution of protein-bound sulfhydryl groups has been noticed: low in the basal, moderate in the granular and negative or very low in the cornified layer. This was fully confirmed in the present studies. The histochemical findings are consistent with the present biochemical assumption[14)] that the epidermal keratinization is a continuous process occurring throughout the Malpighian layer. The diminution of sulfhydryl groups in the cornified layer has been assumed to result from the oxidative conversion into disulfide bonds, which takes place as the last stage of the chemical processes in keratinization.

Recently, autoradiographic examination has come to be used for the study of keratinization. In 1954, Bern *et al.*[3),4)] reported an interesting fact that S^{35}-labeled cystine, when injected to animals, was rapidly incorporated into the keratinizing tissues, especially into the keratogenous zone of hair-follicle and conical papilla of the tongue. This was confirmed and extended by several authors,[4),7),15),16)] using S^{35}-labeled cystine or methionine. It has been emphasized that the specific uptake of radio-cystine was most prominent in the area above the zone of active proliferation and negative or very slight in the basal cells. The present findings were in full agreement with these observations, and it was also shown that in the skin as well as in the tongue and esophagus the incorporated cystine-S^{35} went within 72 hours almost completely over to the cornified layer.

Prominence of Barrnett-Seligman's reaction for sulfhydryl groups in the granular layer was remarkable in the author's study, and this was paralleled by the exceedingly high incorporation of the radio-cystine. This apparently supports the view that the role played by the granular layer is important in the whole process of keratinization. Although, Marston (1938[11)]) and Rothman (1954[14)]) suggested the possibility of unfolding of the polypeptide chains as the cause of the high content of sulfhydryl group in this layer, the present autoradiographic findings may indicate that this is rather concerned with an active progress of protein synthesis there which includes the uptake of S-containing amino-acids from without. We have as yet, however, no information concerning the pathway of the material.[4)]

B. Pathologic keratinization:

1. Parakeratosis: In the present studies, the parakeratotic area was associated with high activity of sulfhydryl groups as revealed by the histochemical reaction, and the portion adjoing the granular layer showed an intense radioactivity 24 hours after the cystine-S^{35} administration, although negative at 8 hours. The findings were constant both in the benign acanthosis and in neoplastic lesions, and believed to represent incompleteness of the keratinizing process on one hand and elevated activity of cell proloferation on the other.

2. Hyperkeratosis: The hyperkeratosis, observed histologically, appeared to include two types of the property in sulfhydryl histochemistry. The one showed an intensive reaction of SH-histochemistry as in the case of parakeratotic portion. The other was low positive in the reaction. The latter may correspond to the ichytiotic type as reported by Rothman[14] (1954) and Van Scott[17] (1954).

C. Keratinization in the epidermal tumor:

In the undifferentiated type of epidermal carcinoma, the cytoplasma of the malignant cells were low both in the SH-content and radio-cystine uptake. This was quite comparable with the attitude of the basal cells of the epidermis in normal and benign conditions. In contrast, the tumor cells of the differentiated type of epidermal carcinoma revealed the feature of the spinous cell of the normal squamous epithelium.

Eisen and Montagna[8] (1954) examined experimental epithelioma in mice by means of Barrnett-Seligman's method, reporting that in the epithelioma the parakeratotic portions were histochemically indistinguishable from the granular layer. The present results, however, revealed that the two are completely different in the biological behavior. Whereas the granular layer showed remarkable blackening in autograph at 3 hours after injection of cystine-S^{35}, the parakeratotic layer showed it never before 8 hours had elapsed.

All of the above observations suggest that there is no essential qualitative difference in the process of keratinization between the neoplastic and normal epidermis, as well as the benign hyperplastic epidermis. Although the squamous carcinoma presents a variegated and even apparently abnormal pictures in the sulfhydryl histochemistry and in the cystine-S^{35} autoradiography, the variation can easily be understood on the basis of proliferative activity as revealed in the ordinary histological pattern of the neoplasma, and this should not be regarded as essential deviation from the normal. It is suggested also that the grade of differentiation of the epidermal tumor can be regarded as expressed in the grade of keratinization of its cells.

Summary

1. Correlative histochemical and autoradiographic study was made on the keratinization in the experimental epidermal tumor, hyperplastic epidermis and normal keratinizing tissues.

2. No essential qualitative difference was noticed between the epidermal tumor and normal keratinizing tissues.

3. The grade of differentiation of the epidermal tumor may be regarded as expressed in the grade of keratinization of its cells.

The present study was carried out under the guidance of Prof. Kunio Oota, Cancer Institute, Tokyo, and Pathology Department of Tokyo Medical & Dental College, to whom my thanks are

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Explanation of Figures (Plates IV~VII)

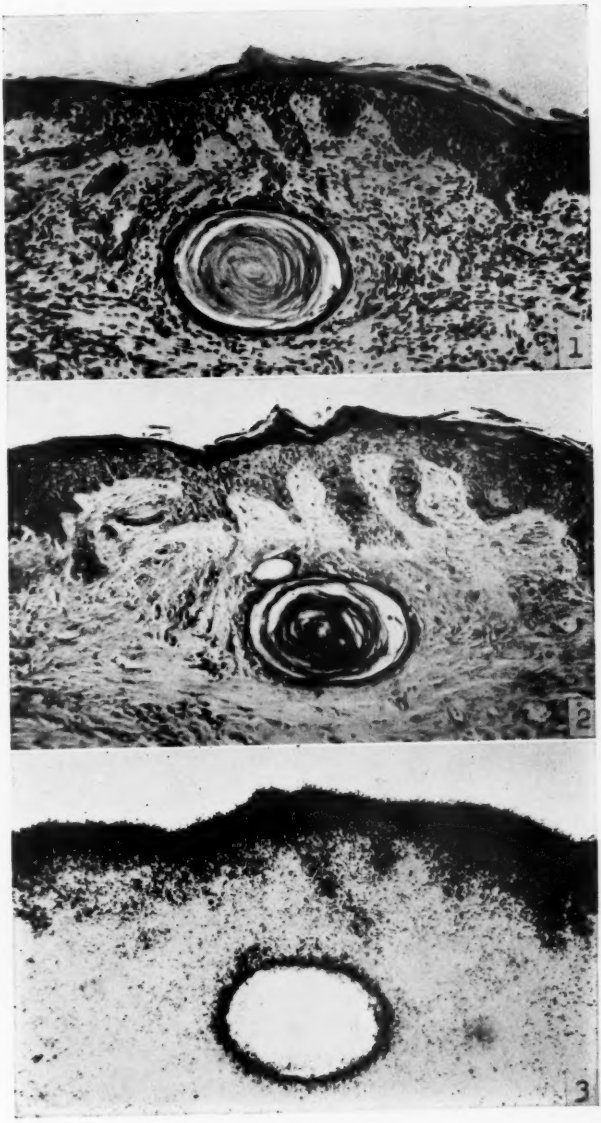
Figs. 1, 2, 3: (Group-I, 9) Hyperplastic epidermis and epidermoid cyst. 100X. 1: HE. 2: DDD. The granular and cornified layers show an intensely positive histochemical reaction. The spinous and basal layers are moderate and low respectively in the reaction. 3: Autoradiograph, 24 hours after the cystine-S^{35} injection. A prominent blackening is noticeable in the granular layer and the lower portion of the cornified layer bordering the granular layer.

Figs. 4, 5, 6: (Group-I, 12) Pseudoepitheliomatous hyperplasia, 100X. 4: HE. 5: DDD. 6: Autoradiograph, 8 hours after the injection of cystine-S^{35}. The cornified layer shows an intense histochemical reaction but is not radioactive.

Figs. 7, 8, 9: (Group-I, 7) Papilloma, 200X. 7: HE. Notice the conspicuous hyper-parakeratosis. 8: DDD. The histochemical reaction is intensely positive in the cornified area. 3: Autoradiograph, 8 hours after the cystine-S^{35} injection. No radioactivity is noticeable in the cornified layer.

Fig. 10: (Group-I, 10) Keratoacanthoma, 10X. Autoradiograph, 8 hours after the injection of cystine-S^{35}. The central Keratin mass shows no radioactivity.

Figs. 11, 12: (Group-I, 13) Squamous cell carcinoma, 200X. 11: DDD. 12: HE. Horn pearls are rich in SH-content. The cytoplasma of differentiated cells (left) shows a moderate histochemical reaction. The cytoplasma of undifferentiated cells is low in the reaction.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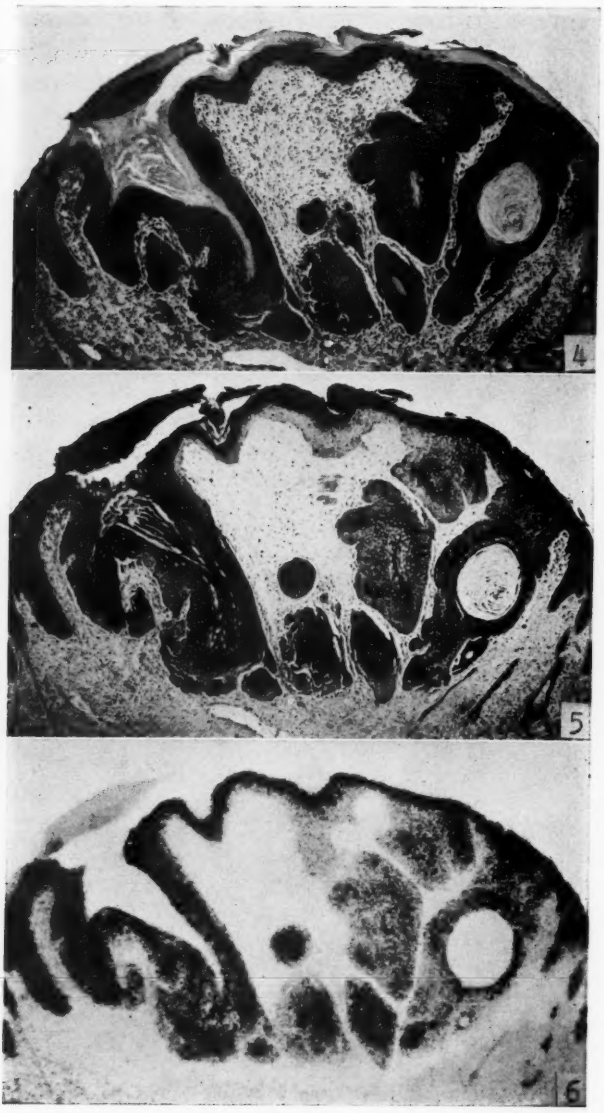
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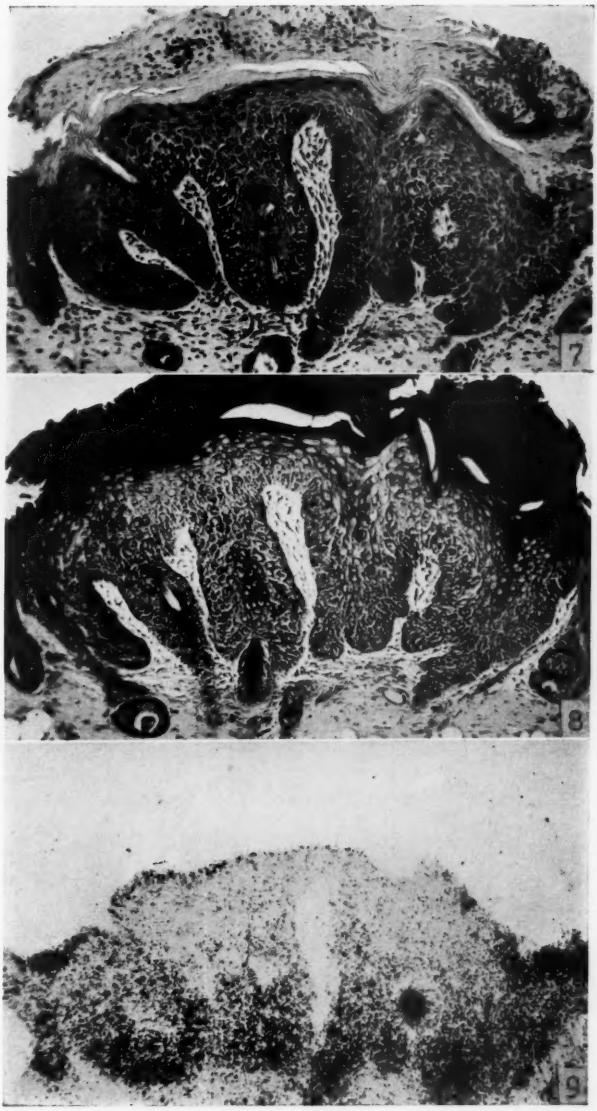
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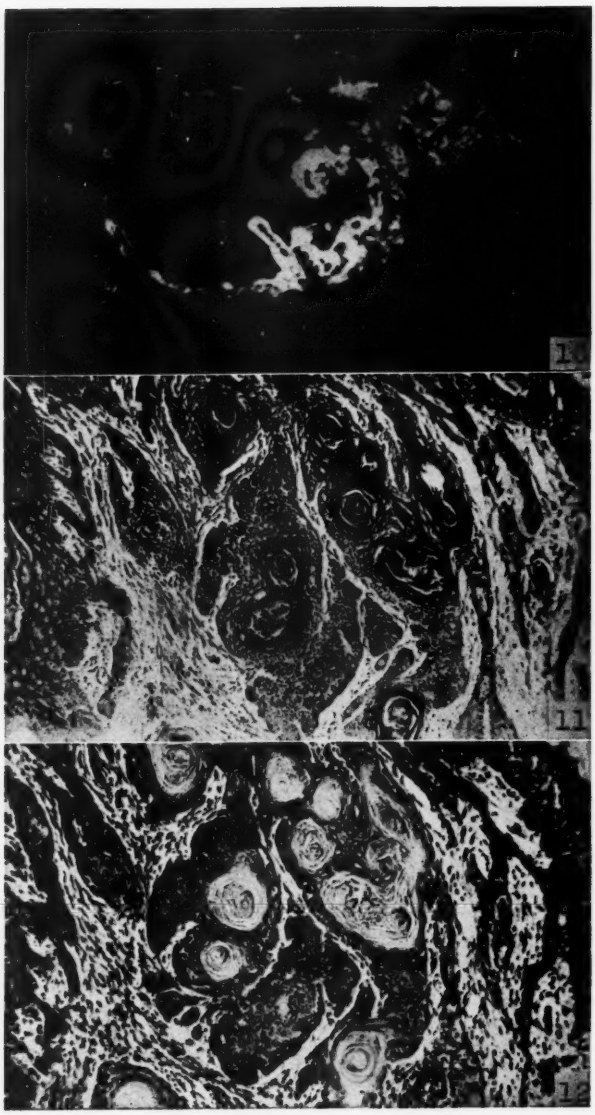
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[GANN, 51, 113~118; March, 1960]

STUDIES ON THE DYE-BOUND PROTEINS FROM THE LIVER OF RATS FED THE AMINOAZO DYES. I

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Following the discovery of the chemical binding between carcinogens and cellular proteins, several people[1),2),3)] have investigated the chemical structure of the binding. The nature of a protein or proteins responsible for the binding, however, has not been investigated except in some works mostly done by Sorof and his coworkers[5),5)] and Arcos.[6)]

Miller and Miller have postulated the idea[7)] that the carcinogenic azo dyes bind with a key protein contolling the cellular growth. As a result of the binding, followed by the delection of the corresponding protein, the cells which have become free from growth controlling mechanism of the body, i. e. cancerous cells, are imagined to arise.

The azo dye binding among subcellular fractions prepared immediately after the administration of the labelled azo dye was studied by Hultin,[8),9)] who observed that the specific amount of protein bound dye was greatest in the microsome fraction among the subcellular fractions at the begining and later the soluble supernatant fraction surpassed the microsome. Hultin[8),9)] and Miller & Miller[10)] have assumed that the binding may take place in the course of the protein biosynthesis in the microsome. Terayama[11)], however, has presented another idea that the microsome is the main locus where the azo dye is metabolized and the binding arises in the course of the dye metabolism.

Sorof et al.[4)] made electrophoretic and ultracentrifugal analysis of the proteins in the soluble supernatant fraction from rats liver given azo dyes. They discovered that most of the protein bound dye is localized in a fraction named "h" fraction, which is sedimented and migrated slowly. Sorof et al.[12),13)] recently subfractionated the "h" fraction and found the protein bound dye to be localized in the two subfractions called slow h_2 and h_3. Recently Arcos et al.[6),14)] tried the starch zone electrophoresis of the supernatant fractions of liver homogenate of rats fed the azo dyes, and showed the presence of two bands moving towards the cathode at pH 7.4 (0.02M barbital buffer). The fast moving, main band seems to correspond to Sorof's so-called "h" fraction. No protein bound dye was observed in the fractions moving towards the anode, according to Arcos.

The experiments were carried out to fractionate the dye bound protein from the homogenate or the supernatant fraction. The experimental results obtained up to the

present, however, seem to sugest that the protein responsible for the azo dye binding is not a single, specific protein, but many, somewhat non-specific proteins which are distributed among the supernatant soluble proteins of rat liver.

Experimental Procedure

Administration of Azo Dyes: Five to six male albino rats weighing 150g were used. 25mg of 3'-Me-DAB or other aminoazo dyes dissolved in one ml of olive oil was administered with the aid of a stomach tube except in cases where the rats were fed on a rice diet containing 0.06% 3'-Me-DAB.

Preparation of the Liver Homogenate: The rats were sacrificed by decapitation. The liver was perfused in situ with the physiological saline solution, then removed immediately and homogenized. A Waring blendor homogenizer was used for two minutes at 0°C for the purpose. Usually 30% liver homogenate in 0.25 M sucrose was prepared, and used for further fractionations.

Acetone Fractionation: Acetone fractionation was carried out in a cold bath regulated at −5°C to −10°C.

Starch Zone Electrophoresis: The 30% acetone supernatant was separated by centrifuging at 10,000 rpm for 30 min. at −7°C to −8°C. The supernatant was dialysed against running water, then against 15 to 20% polyvinylpyrollidone (pH 7.0). The concentrated protein solution in a dialysing cellophane bag was then lyophylized or immediately subjected to the zone electrophoresis. A 1.5×5×45 cm plastic trough was used. The general condition of the electrophoresis was as follows:

pH: 7.9 (0.02 M barbital buffer containg 0.055 M KCl). Voltage: 200-240V. Current: 25 to 30mA. 40 to 45 hours of running in a cold room (near 0°C).

After the electrophoresis the starch column was divided into many blocks by cutting with two cm width. The protein was extracted from every block with pH 7.4, 0.004M phosphate buffer containing 0.026 M KCl in three runs, using about 30 ml altogether.

Estimation of Polar Dyes: A great portion of the protein extract was heated. After cooling off, the *Streptomyces griseus* protease was added and the mixture was incubated at 37°C for several days under toluene untill the protein coagulate disappeared. After the solution was washed with benzene, the polar dye (peptide bound azo dye) was extracted with n-butanol. The butanol was evaporated under reduced pressure and the residue was dissolved in 2N HCl. The HCl solution was washed with ethylacetate two or three times. The absorption spectra were obtained with thus partially purified polar dye solutions in 2N HCl (600mμ-360mμ). The amount of the polar dye was calculated as in the preceding study in term of DAB.

Estimation of Proteins: A one-tenth or two-tenth ml portion of the protein extract was mixed with 3 ml of the cupper solution having the composition of 50 parts of 2% sodium carbonate in 0.1N and one part of 1% $CuSO_4 \cdot 5H_2O$, 2% potassium tartarate,

and was kept at room temperature for 15 min. Three-tenth ml of Folin's solution was added and the optical density at 660mμ was measured after 30 to 50 minutes. The amount of protein was expressed in terms of optical densities times volume, unless it is measured by the micro-Kjeldahl technique.

Results and Discussions

Acetone Fractionation: The 30% liver homogenate was subjected to the acetone fractionation. At every stage of the following acetone concentrations, 10%, 30%, 50%, and 80%, the precipitate was separated from the supernatant by centrifuging at 5,000 to 10,000 rpm for 15 to 40 minutes at low temperature. The precipitates were dissolved in 20 to 30ml of 0.1M sodium acetate, heated and subjected to the proteolysis for the estimation of the polar dye. A small portion was subjected to the micro-Kjeldahl measurement for protein estimation. The result is shown in Table 1.

Table 1. The Distribution of the Protein bound Azo Dye and Proteins among the Fractions by the Acetone Fractionation of Rats Liver Homogenate (3'-Me-DAB 25 mg, 50 g Liver)

	Polar Dye (γ)	Protein (g)	Polar dye/Protein
Whole Homogenate	48.3 (100%)	4.44	10.8
0–10% Ppt.	8.5 (17.6%)	(1.88)	4.5
0–10% Sup.		2.56	
10–30% Ppt.	6.0 (12.4%)	(1.57)	3.8
10–30% Sup.		0.99	
30–50% Ppt.	14.1 (29.2%)	(0.49)	28.6
30–50% Sup.		0.50	
50–80% Ppt.	10.4 (21.5%)	(0.24)	43.3
50–80% Sup.	8.0 (16.5%)	0.26	30.8

Ammonium Sulfate Fractional Precipitation of 30% Acetone Supernatant: 30% rat liver homogenate (50g liver) was mixed with cold acetone up to the acetone concentration of 30% at −7°C to −8°C. Following the centrifugation, the 30% acetone supernatant was dialysed against running water for 23 hours. The dialysate (206 ml) was submitted to the fractional precipitation by adding powdered ammonium sulfate. The precipitates were separated by centrifugation at the following degrees of ammonium sulfate saturation, 0.4, 0.5, 0.6, 0.7, 0.8 and 1.0 S. The precipitates were dissolved in 10 to 15 ml of pH 7.4 phosphate buffer and dialysed against running water for 24 to 40 hours at 4 to 5°C. Micro-Kjeldahl estimation of nitrogen and the polar dye estimation were carried out with every fraction. The result is shown in Table 2.

Table 2. Distribution of Protein bound Dye and Proteins among Fractions by Ammonium Sulfate from 30% Acetone Supernatant of Rats Liver Homogenate. (3'-Me-DAB 25 mg, 50 g Liver)

Fractions	Polar Dye (γ)	Protein (g)	Polar dye/Protein
Whole Homogenate	81.3	8.38	9.7
0–0.2 S Ppt.	0.5	0.032	15.6
0.2–0.4 S Ppt.	12.1	0.269	45.0
0.4–0.5 S Ppt.	5.9	0.285	20.7
0.5–0.6 S Ppt.	10.8	0.313	34.5
0.6–0.7 S Ppt.	14.7	0.330	44.6
0.7–0.8 S Ppt.	11.6	0.230	50.5
0.8–1.0 S Ppt.	5.7	0.142	40.1
1.0 S Sup.	0.5		

Starch Zone Electrophoresis: The procedure of the electrophoresis is described above. As the material, 30% acetone supernatant, dialysed and concentrated, was used except in the case of No. 6 where the ultracentrifugal supernatant from the homogenate was used. The relative amount of proteins in every block was estimated by Folin's method. The following cases different in dyes and administration methods were investigated (Table 3).

Table 3.

Exp. No.	Dyes	Administration Method	Protein Bound Dye (γ/gProtein)
1	3'-Me-DAB	25 mg, stomach tube, 2 days later	50
2	3'-Me-DAB	0.06% rice diet, 14 days	50
3	3'-Me-DAB	0.06% rice diet, 30 days	43
4	2-Me-DAB	50 mg, stomach tube, 2 days later	25
5	AB	50 mg, stomach tube, 2 days later	21
6	3'-Me-DAB	25 mg, stomach tube, 2 days later	10

The zone electrophoretic patterns sbserved in the above experiments are illustrated in Figs. 1–6, which correspond to Exp. No. 1–6 in Table 3 respectively.

General Discussion: The precipitate at 30% acetone concentration from the homogenate occupies about 77% of the total protein of the homogenate, while only 30% of the total protein bound dye is found in this part. The supernatant at 30% acetone therefore contains 70% of the total protein bound dyes. This amount seems to be a little larger compared with the protein bound dye in the ultracentrifugal supernatant (50 to 60%) and might contain some part of the protein bound dye from other cellular components, for instance, from microsome.

It is very interesting to see that the dye-binding proteins in rats liver are rather soluble even in high acetone concentrations as shown in Table 1. The ammonium

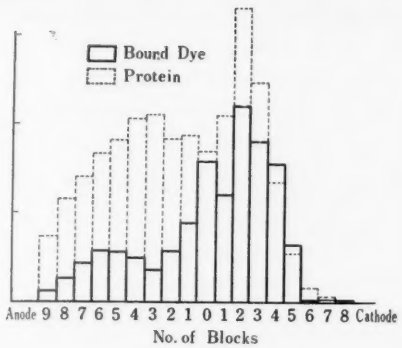

Fig. 1

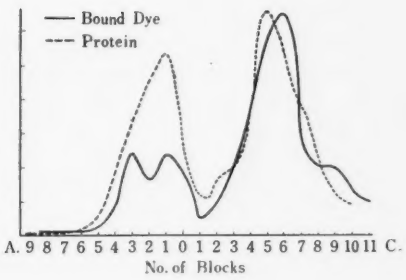

Fig. 2

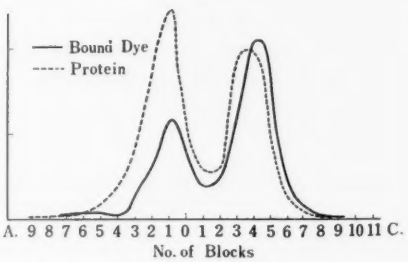

Fig. 3

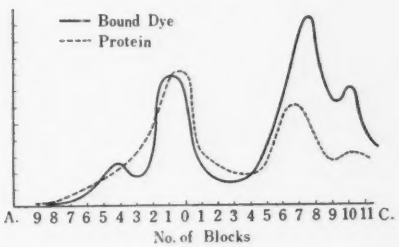

Fig. 4

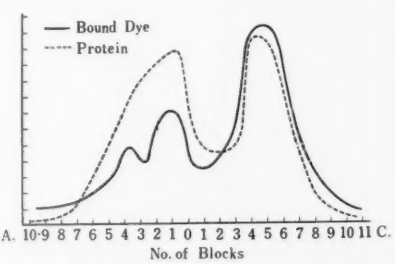

Fig. 5

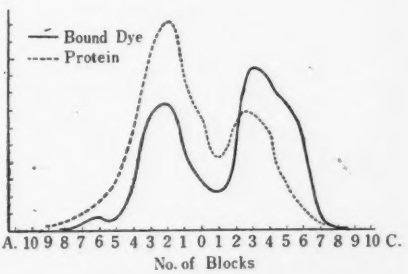

Fig. 6

sulfate fractional precipitation clearly demonstrates that there are at least two groups of dye-binding proteins in 30% acetone supernatant, one precipitated at 0.5-0.4 S and the other at 0.7-0.7 S.

The starch zone electrophoresis of the soluble proteins of rats liver fed various kinds of azo dyes (carcinogenic or noncarcinogenic) demonstrated qualitatively similar electrophoretic patterns with respect to the protein bound dye migrations. The majority of the protein bound dyes migrates towards the cathode. The minor part moves towards the anode. In most cases the third band is observed in the vicinity of the original place or a little toward anodic side. The first peak having a rather

big negative mobility occupies 70% of the total protein bound dyein the 30% acetone supernatant and seems to correspond to the "h" fraction presented by Sorof et al. The Arcoses have also observed this in their zone electrophoretic experiments but they failed to find the minor bands migrating towards the anode.[6),14)] Even though there is no qualitative difference among the electrophoretic patterns for the various dyes described above, a considerable difference is observed in the quantitative sense as shown in Table 3. The noncarcinogenic dyes such as AB or 2-Me-DAB show rather small protein binding compared with 3'-Me-DAB, a potent carcinogen.

Summary

1) Acetone and ammonium sulfate fractionations were carried out on the rats liver homogenates administered with 3'-Me-DAB.

2) The starch zone electrophoretic patterns were compared among the supernatants of rats liver homogenate fed carcinogenic 3'-Me-DAB and non-carcinogenic 2-Me-DAB or AB. No significant difference was observed in the patterns but the amount of the protein bound dye was greater with the carcinogenic azo dye than with the noncarcinogenic ones.

3) At least three protein groups responsible for the dye binding seem to exist in the supernatant from the electrophoretic pattern. The general impression from the comparison of the electrophoretic pattern of the bound dye with that of the protein, however, does not support the presece of any specific protein responsible for the binding but may indicate more or less random binding, even though not completely evenly among the various kinds of liver proteins.

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